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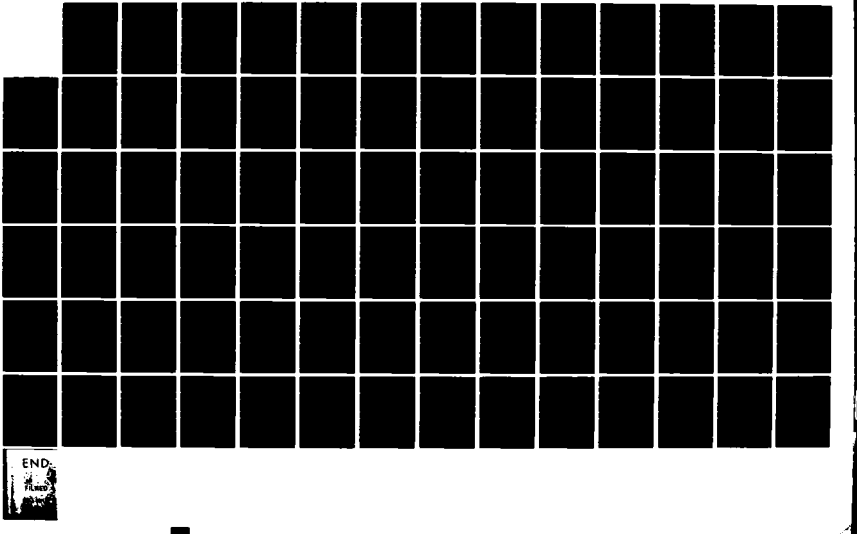

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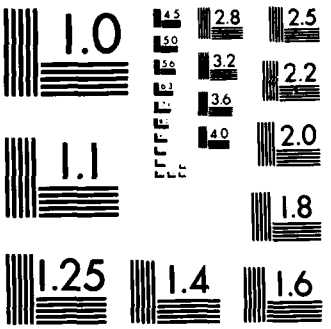


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# IMMUNOLOGIC CONTROL OF DIARRHEAL DISEASE DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI: REACTOGENICITY, IMMUNOGENICITY, AND EFFICACY STUDIES OF PURIFIED ESCHERICHIA COLI H10407 TYPE 1 SOMATIC PILI VACCINE

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ANNUAL REPORT

September, 1980

Prepared by:

Myron M. Levine, M.D., D.T.P.H., Principal Investigator

Robert E. Black, M.D., M.P.H., Co-Principal Investigator

Mary Lou Clements, M.D., M.P.H., Associate Investigator

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Center for Vaccine Development, Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, Md. 21201

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## BACKGROUND

In 1978, in studies supported by the U.S. Army Medical Research and Development Command, we demonstrated by means of volunteer challenge studies that clinical diarrheal infection due to enterotoxigenic Escherichia coli (ETEC) strains B7A (O148:H28)[1] or H10407 (O78:H11)[2] stimulated immunity to re-challenge with the homologous strain. Although clinically protected, re-challenge volunteers excreted the challenge organism to the same quantitative degree as ill controls.[1,2] We hypothesized at that time that the non-bactericidal mechanism of immunity was operative at the intestinal mucosal surface and involved prevention of adherence of ETEC to mucosa of the small intestine. We suggested a role for vaccines consisting of purified adhesion pili. The striking success of purified K88, K99 and 987P purified pili vaccines in veterinary studies[3-6] provided a notable precedent for optimism regarding the value of pili vaccines in preventing ETEC disease in man.[7]

In the 1979 contract year we focused on two main tasks:

1) Establishing the frequency with which CFA/I, CFA/II and type 1 somatic pili were encountered in ETEC strains of known virulence (i.e. employed in volunteer studies[8]) and in strains freshly isolated from cases of acute Travelers' Diarrhea throughout the world.[9]

2) Carrying out the first studies in man evaluating the safety, immunogenicity and efficacy of E. coli purified type 1 somatic pili vaccine.*

We found that a number of ETEC strains that lack CFA/I or CFA/II, nevertheless caused diarrheal disease when fed to man.[8] Similarly, preliminary analysis of a series of fresh ETEC strains from cases of Travelers' Diarrhea throughout the world (kindly provided by Dr. R.B. Sack) revealed that only a minority of the strains possessed CFA/I or CFA/II.[9] Many strains lacking CFA/I or II did have type 1 somatic pili. This observation raised the hope that perhaps vaccine-induced antibody to type 1 somatic pili could protect against ETEC strains having

*See Appendices A and B.

this antigen and lacking CFA/I and II.

Preliminary studies in the 1979 contract year with parenteral purified type 1 somatic pilus vaccines provided the following observations:

1) No adverse reactions were encountered in volunteers given a single intramuscular inoculation with 45, 90, 900 or 1800 mcg of purified pili vaccine.

2) Pili vaccines did not alter intestinal transit time (measured with Carmine Red dye), intestinal absorption (evaluated by D-Xylose tests), or the prevalence of normal colonic flora E. coli that possess type 1 somatic pili of the H10407 antigenic variety.

3) All persons developed significant rises in circulating pili antibody (measured by ELISA) following primary immunization; antibody levels increased with vaccine dose.

4) Pili vaccine did not stimulate rises in LT antitoxin but trace amounts of O antigen contained in the vaccine led to significant rises in O antibody in all 10 recipients of the 1800 mcg primary dose and in one-half of those given lower doses.

5) Fifteen persons received an 1800 mcg booster dose of vaccine 28 days after primary. Six of these volunteers developed local adverse reactions (heat, induration, erythema or tenderness) which in all but one instance lasted less than 48 hrs.

6) One month after booster inoculation, six vaccinees who received two doses of purified pili and 7 unimmunized control volunteers were challenged with $5X10^8$ virulent H10407 organisms in a challenge study of vaccine efficacy. Diarrhea occurred in all 7 controls, including copious cholera-like purging in three persons (of whom two required intravenous rehydration). Malaise occurred in all controls and vomiting in 6 of 7. In contrast, only 2 of 6 vaccinees developed diarrhea ($p=0.04$) and neither manifested malaise, vomiting or fever.

7) Despite clinical protection, all vaccinees as well as controls excreted

E. coli H10407. Of 602 H10407 clones from coprocultures examined during challenge, 100% possessed both type 1 somatic and CFA/I pili.

WORK ACCOMPLISHED DURING 1980 CONTRACT YEAR

Based on the above-mentioned observations made in the 1979 contract year, our priority tasks in the 1980 contract year were as follows:

1) To develop an immunogenic, non-reactogenic two dose parenteral immunization schedule that would stimulate brisk anti-pili antibody responses without causing adverse reactions. With development of such an immunization procedure, further efficacy challenge studies could proceed.

2) Challenge studies to assess the efficacy of the new, non-reactogenic two-dose immunization schedule.

3) More detailed investigation of the circulating O antibody response noted to occur following primary immunization of volunteers with type 1 somatic pilus vaccine.

4) Further studies to assess the frequency of occurrence of CFA/I, CFA/II and type 1 somatic pili in ETEC pathogens from diverse geographic sources.

Development of a Non-Reactogenic Immunogenic Two-Dose Immunization Schedule for Type 1 Somatic Pilus Vaccine

Reactogenicity:

Twenty-one volunteers were inoculated IM with 900 mcg of purified pili vaccine. Twenty-eight days later, 11 received booster inoculation with 450 mcg, while the other 10 got 180 mcg. With each vaccination an equal volume of physiologic saline was inoculated into the contralateral arm. Evaluation at 24 and 48 hrs. was double-blind: neither volunteer nor interviewer knew which inoculation contained vaccine and which was saline. No adverse local reactions were observed following primary or booster immunization. No volunteer experienced nausea, vomiting or change in stool pattern and all maintained normal D-Xylose absorption tests when tested 28 days after primary and booster immunizations.

Immunogenicity:

Sera collected from volunteers on days 0, 10, 21, 28, 42 and 56 relative to primary immunization were tested for:

1) circulating IgG anti-type 1 somatic pili antibodies by ELISA technique;

2) circulating LT antitoxin by Y-1 adrenal cell neutralization technique;

3) circulating O antibody by passive hemagglutination technique utilizing purified H10407 lipopolysaccharide O antigen adsorbed to glutaraldehyde-treated sheep erythrocytes.

All 20 immunized volunteers developed four-fold or greater rises in anti-pili antibody following primary immunization. The mean seroconversion ratio of titers on day +28 over the baseline was 32, evidence of a brisk immune response.

The kinetics of the immune response in the two groups of vaccinees is shown in Figure 1. Geometric mean titers (GMT) on day 28 were similar in both groups. One month following the booster inoculation (day +56), the GMT was slightly, but insignificantly, higher in the group who received 450 mcg boosters.

No vaccinees developed significant rises in anti-LT antitoxin by Y-1 adrenal cell assay.

Nine of the 21 volunteers (43%) developed four-fold or greater rises in O antibody following primary immunization; in all but one instance O antibody peaked on day +10. Five additional vaccinees had two-fold rises in O antibody after the first dose of vaccine which also were seen on day +10. The kinetics and magnitude of the O antibody response in the two groups of vaccinees are shown in Figure 2.

In Table 2 the GMTs of volunteers immunized by the modified two-dose schedules are compared with eight volunteers from the preliminary studies carried out in 1979. The persons immunized in 1979 received an 1800 mcg booster following primary immunization with 900 or 1800 mcg. One can see that the GMT increases somewhat with size of the booster dose. Considerable variability occurs, however. In

Table 3 are shown the distribution of day +56 titers in the three groups of vaccinees. One can see that even in the group that received 1800 mcg boosters, three of eight vaccinees had reciprocal titers 100 or less and two were below 50. One must bear in mind that the 1800 mcg booster inoculation in the 1979 studies caused unacceptable local adverse reactions.

One observation of interest that may have relevance as we progress in these studies is that only 43% of the persons immunized by the new two dose schedule had significant rises in anti-O antibody versus all individuals in the 1979 studies who received an 1800 mcg primary vaccine dose.

Efficacy Challenge Studies of Volunteers Immunized by the Non-Reactogenic, Immunogenic Vaccination Schedule

The 900/450 mcg two dose immunization schedule was selected for further studies since it gave slightly higher antibody levels than the 900/180 mcg schedule (Table 2, Figure 1), while being equally well-tolerated. A group of six volunteers immunized by the 900/450 mcg schedule participated in a challenge study of vaccine efficacy along with eight unimmunized control volunteers. Following inoculation with $5X10^8$ virulent H10407 organisms, seven of eight controls developed diarrhea. Four of the seven controls purged more than two liters. As seen in Table 4, malaise, fever and vomiting were common. In this challenge three of six vaccinees also developed diarrheal disease with no evidence of attenuation of clinical illness. One vaccinee purged 10.6 liters in 31 stools; fever, malaise and vomiting were also seen in the ill vaccinees (Table 4). In this challenge vaccine efficacy was only 43% and the difference in attack rates between the groups (3/6 vs. 7/8) was not significant.

Several possible explanations come to mind to account for the diminished vaccine efficacy observed in this challenge study compared to the 1979 challenge study:

1) The slightly lower levels of circulating IgG antipili antibody resulting

from the non-reactogenic two-dose immunization schedule compared with levels resulting from the reactogenic higher dose schedule used in 1979 may have diminished efficacy. Since ETEC are non-invasive enteropathogens against which mucosal immunity is probably paramount, one can imagine that a parenteral vaccine would have to stimulate extremely high levels of circulating antibody to allow sufficient antibody to leach onto the mucosal surface to mediate protection.

2) It is conceivable that the more prominent O antibody response seen in vaccinees who received 1800 mcg doses of pili vaccine in 1979 played a synergistic role in enhancing protection against a high challenge dose of the homologous strain in the 1979 challenge. The lower O antibody response that followed the non-reactogenic lower dose immunization schedule used in the current trials may have precluded synergism.

3) Both in the 1979 and the first 1980 vaccine efficacy challenge studies, relatively high inocula ($5X10^8$) of a cloned H10407 organism were fed to volunteers, resulting in extremely high attack rates in controls (100% in 1979, 88% in 1980) and characterized by severe diarrhea. Large inocula were employed because of the relatively small numbers of volunteers involved. Also a small homologous re-challenge study with E. coli H10407 had shown that prior clinical illness protected against re-challenge with an inoculum of $10^8$ organisms causing diarrheal illness in 75% of controls.[2]

It is conceivable that such high inocula comprising clinical $ID_{90-100}$ are unnaturally high and represent unrealistic overwhelming challenge inocula that can overcome vaccine-induced immunity.

4) One could argue that although the purified type 1 somatic pili were prepared from ETEC strain H10407, this is an inappropriate organism to employ as a challenge strain since it also bears CFA/I pili,[8,10] another potent adhesion for mucosal cells. Rather, one could argue, challenge studies should be carried out employing

ETEC strains having the H10407 antigenic variety of type 1 somatic pili but lacking CFA/I and II or other mannose-resistant hemagglutinins.

It is likely that all four of the above explanations played some part in the results observed. In order to assess the relative importance of the size of the challenge inoculum, a second challenge study is scheduled to be completed in September, 1980 wherein a group of volunteers immunized by the 900/450 mcg schedule will be challenged with $10^7$ H10407 organisms, representing an $ID_{30-40}$. A large number of controls is being included in this study.

The possible confounding role of O antibody in synergistically enhancing protection of pili vaccines against homologous H10407 can be ruled out by means of the heterologous studies using strain B7A proposed for next year. E. coli B7A (O148:H28) differs in its O and H antigens but possesses type 1 somatic pili that are antigenically identical to those of H10407. B7A lacks CFA/I or II.[8] Therefore, with B7A challenge we have an ideal system to assess the protective efficacy of vaccine-induced antibody to type 1 somatic pili.

Intensive Investigation of the O Antibody Response following Immunization with H10407 Type 1 Somatic Pili Vaccine

In the preliminary dose response studies with type 1 somatic pili vaccine carried out in 1979 it was found that one-half the individuals inoculated with 45 to 900 mcg and all inoculated with 1800 mcg single doses of purified pili developed four-fold or greater rises in O antibody measured by passive hemagglutination technique[2,11] using a heated alkaline hydrolysate O antigen[12] prepared by Charles Young at the Center for Vaccine Development. The absence of adverse reactions seen after primary immunization, even in recipients of 1800 mcg, suggested that the level of residual O antigen in the vaccine preparation must be minuscule; i.e. sufficient to stimulate an antibody response but not to cause overt clinical reactions. An alternative explanation was that the heated alkaline hydrolysate O antigen prepared

at the CVD (henceforth referred to as CVD) may have been contaminated by type 1 somatic pili which would account for the rise in titers. The CVD O antigen, however, was prepared from a solid agar-grown clone of H10407 shown to lack type 1 somatic and CFA/I pili by hemagglutination tests and agglutination with specific antisera. In order to rule out the latter possibility and to clarify the situation, a series of laboratory studies were carried out which will be summarized below.

Limulus Assay

A sample of E. coli H10407 type 1 somatic pili vaccine from the vial used to inoculate volunteers was tested for presence of endotoxin by the Limulus gel assay. The vaccine was found to be quite positive. However, the specific identity of the endotoxin source could, of course, not be ascertained.

Testing of Sera from H10407 Challenge with CVD Antigen

Of the 13 individuals (6 vaccinees, 7 controls) who participated in the 1979 challenge study, none developed rises in circulating antibody to type 1 pili following challenge (see Table 4 of Appendix B). However, using the CVD antigen (undiluted) adsorbed to glutaraldehyde-treated[13] sheep erythrocytes as described on p.3-4 of Appendix B, all individuals who ingested virulent H10407 developed four-fold or greater rises in O antibody titer (Table 5). Antibody peaked on day +10 post-challenge, characteristic of the classic IgM O antibody response that follows enteric infection.[14]

Testing of Sera of Vaccinees with E. coli B7A Heated Alkaline Extract Antigen

E. coli B7A (O148:H28) has distinct O and H antigens from E. coli H10407 (O78:H11) but possesses the identical antigenic variety of type 1 somatic pili. Therefore a heated alkaline extract O antigen was prepared from a clone of B7A that was shown to be highly piliated (by mannose-sensitive hemagglutination of guinea pig erythrocytes and agglutination with type 1 pili antiserum). This clone was cultured for 48 hrs. in stagnant Mueller-Hinton broth to further enhance

type 1 piliation. Thus if type 1 somatic pili were indeed contaminating the heated alkaline hydrolysate antigen preparations, vaccinees who developed rises against the CVD 078 antigen should also show rises against the 0148 antigen. Results of PHA tests for O antibody using both antigens are shown in Table 6. None of the vaccinees who had significant rises against H10407 antigen had rises against B7A antigen. This strongly suggested that pili contamination of the CVD antigen was not responsible for the antibody titer rises manifested by the vaccinees.

Serologic Tests with an LPS Antigen Prepared by Brinton's Laboratory

In order to further clarify the situation regarding O antibody responses, a more highly purified H10407 O antigen preparation was made by the butanol extraction method[15] by Peter Fusco in C.C. Brinton's laboratory in Pittsburgh (henceforth referred to as the Pittsburgh H10407 O antigen). This antigen was diluted 1:300 (10 mcg/ml) and adsorbed to sheep erythrocytes for PHA tests.

Sera from the 21 pili vaccinees were tested concomitantly using both Pittsburgh and CVD antigens at 1:300 dilution. These results are shown in Table 7 along with titers of these sera run against undiluted CVD antigen. Titers of sera tested with neat or 1:300 diluted CVD antigen correlated closely (Figure 3). The rates of seroconversion were identical whether Pittsburgh or CVD (1:300) antigen were used. In Figure 4 is seen the correlation between PHA titer obtained using Pittsburgh or CVD (1:300) antigen. The coefficient of correlation is 0.89 ($p<0.001$). These data demonstrate that the PHA serologic results are unchanged whether one uses highly purified Pittsburgh (butanol-extract) or CVD (heat alkaline hydrolysate) antigen. Obviously, pilus contamination of the antigen preparations was not responsible for the antibody rises in vaccinees.

O Antibody in Sera of Persons Infected with E. coli B7A Tested by Passive Hemagglutination Using Pittsburgh and CVD H10407 O Antigens

We considered the possibility that the Pittsburgh and CVD antigens contained non-specific E. coli LPS antigens that were reacting with sera from vaccinees and

challenged persons. If so, sera from persons infected with other ETEC serotypes should also have rises to this common or non-specific antigen. Therefore we tested sera from five patients who developed diarrheal disease following challenge with ETEC strain B7A (O148:H28); these sera were tested for O antibody using alkaline extract B7A O antigen and Pittsburgh and CVD H10407 O antigens. As seen in Table 8, while four of five serum pairs showed rises against B7A antigen, no rises were found against either CVD or Pittsburgh antigen. These data further demonstrate the specificity of these H10407 O antigen preparations and the serologic response to them in man.

ELISA Antibody Studies Utilizing Pittsburgh and CVD H10407 O Antigens

Charles Young in the CVD rapidly developed an ELISA test for measuring IgM antibody to E. coli O antigen. Briefly, the method was a modification of his general technique as described for measurement of cholera antitoxin.[16] Pittsburgh or CVD antigen in 1:300 dilution or B7A antigen was applied to every other well of polyvinylchloride microtiter plates. Plates were rinsed with washing buffer and dilutions of test sera were applied to the wells. After appropriate rinses alkaline-phosphatase conjugated goat anti-human IgM was added to the wells, incubated and rinsed. Substrate was added for development of color; the reactions were stopped and plates examined with an ELISA spectrophotometer. Net optical density equals the value when O.D. of a well without antigen is subtracted from the O.D. of its corresponding well containing antigen.

In Table 9 are shown the results of ELISA tests of sera pre- and 10 days post-challenge of volunteers fed ETEC strain H10407. Ten of 13 had rises to Pittsburgh or CVD antigen. Correlation of net O.D. in ELISA tests using the two H10407 antigens is shown in Figure 5.

The specificity of the assay is attested to by results shown in Table 10 wherein no individuals with E. coli B7A infection had rises to H10407 antigens.

Sera from 20 persons immunized with pili vaccine were tested for IgM ELISA antibodies to H10407 O antigens with results displayed in Table 11. The IgM ELISA detected no O antibody rises in individuals who received 45, 90 or 900 mcg doses of vaccine. However, significant rises in IgM O antibody were detected in six of 10 recipients of 1800 mcg using CVD antigen and in four of 10 persons using Pittsburgh antigen. The reason why the IgM ELISA was less sensitive than PHA in detecting O antibody rises in recipients of low doses of vaccine is not entirely clear. This could be due to quality of antibody (i.e. affinity), or technical aspects of the ELISA technique. At any rate, one-half of the recipients of the 1800 mcg vaccine dose had rises by ELISA.

Comment

The exhaustive serologic studies summarized above leave no doubt that a proportion of vaccinees immunized with purified type 1 somatic pili develop rises in O antibody. The level of O antigen contaminating the vaccine preparation is minuscule since it is not sufficient to cause reactions when given as a single dose. These observations lend further import to carry out future studies using E. coli B/A, rather than H10407, as the challenge organism. Such challenges will allow anti-pili immunity to be evaluated without the possibility of confounding effects due to synergy from anti-O antibody.

Further Studies on the Prevalence of CFA/I, CFA/II and Type 1 Somatic Pili in Fresh ETEC Isolates from Diverse Geographic Regions

We continued our studies examining the frequency of occurrence of CFA/I, CFA/II and type 1 somatic pili in ETEC strains from cases of travelers' diarrhea after minimal subcultures and relating their presence to enterotoxin type and serotype. This information, obtained using minimally-passed strains, is critical since the antigenic composition of purified pilus vaccines depends on it and heretofore reports of the prevalence and importance of these antigens in human ETEC have varied greatly.

Bacterial Strains:

ETEC were isolated from Peace Corps volunteers with acute diarrheal disease in the course of travelers' diarrhea studies in Kenya, Morocco, Honduras, Korea, Zaire and Columbia carried out by Dr. R.B. Sack of Baltimore City Hospital. Colonies were confirmed as E. coli by standard biochemical techniques and serotyped by the Orskovs in Copenhagen. Isolates were initially tested for production of LT by Y1 adrenal cell assay and for ST by the infant mouse assay in Dr. Sack's laboratory. Strains that were found to be enterotoxigenic were then transferred to the CVD laboratories to be tested for the presence of pili by hemagglutination and agglutination with antisera.

Cultivation of Strains:

Strains that were found to be enterotoxigenic were cultivated (only 1-3 passages after primary isolation) in a manner to promote piliation. Isolates were cultured both on modified casamino acids/yeast extract (CFA) agar and in Mueller-Hinton broth (15 ml.). CFA agar plates were incubated aerobically for 24 hr at 37°C prior to testing for pili. Mueller-Hinton broth cultures were incubated aerobically for 48 hr at 37°C, subcultured into Mueller-Hinton broth for another 48 hr and tested. The tubes of broth were centrifuged and decanted, and the pellets resuspended with saline to a concentration of $10^{10}$ organisms/ml.

Growth on solid agar (especially CFA agar) is preferential for expression of pili associated with MRHA (such as CFA/I and II), while being less favorable for type 1 somatic pili. On the other hand, static broth cultures offer optimal growth conditions for expression of type 1 somatic pili and sub-optimal conditions for CFA/I and II.

Hemagglutination:

CFA/I and II were identified by MRHA of human type A or bovine erythrocytes, respectively. MSHA of guinea pig erythrocytes detected type 1 somatic pili. Guinea

pig, bovine and human type A erythrocytes were freshly obtained, twice washed in 0.85% NaCl, and divided to prepare 3% suspensions in NaCl and mannose (0.1%); each E. coli strain was tested with all three species of erythrocytes. Hemagglutination (HA) was carried out on glass slides at 24°C and 4°C with human and guinea pig cells and at 4°C with bovine erythrocytes. Several bacterial colonies were harvested with a sterile wooden applicator stick and mixed on the slide with 0.025 ml. of erythrocyte suspension. Agglutination was graded from 0 to 4+, depending on rapidity and strength of reaction.

Agglutination with Specific Antisera:

Antibody to CFA/I and II were prepared by absorption by the method of Evans et al.[10], wherein E. coli strains possessing CFA/I (H10407) or II (A346C1) were used to repeatedly inoculate 2.5 Kg albino rabbits intravenously. The rabbits (two per strain) were exsanguinated after 21 days when the serum agglutinated the immunizing strains at titers ≥1:512. The respective sera were absorbed with E. coli H10407 P (which lacks CFA/I) or E. coli A346C1-P (which lacks CFA/II) until the sera failed to agglutinate these strains. The sera continued to strongly agglutinate H10407 and A346C1-P, respectively.

Results:

Forty diarrheal episodes in forty patients yielded ETEC. In 36 patients infection was apparently due to a single serotype manifesting a single enterotoxin phenotype. Among the remaining four persons, one had ETEC of $LT^+/ST^-$ variety of three different serotypes all sharing the same flagellar antigen (O127(O86):H9, rough:H9, O8:H9) and having the same piliation (type 1 somatic). The other three cases had mixed infections with ETEC of different serotypes, toxin types and piliation.

Of the 36 persons with travelers' diarrhea due to a single ETEC strain, nine infections were due to $LT^+/ST^+$, 13 to $LT^-/ST^+$ and 14 to $LT^+/ST^-$ strains (Table 12).

Overall, seven of the 36 infecting ETEC strains (19%) elaborated CFA/I or II, while 39% (14/36) possessed type 1 somatic pili. There was a distinct relationship apparent between toxin type and piliation. CFA/I or II pili occurred in 4 of nine $LT^+/ST^+$ strains but in none of 14 $LT^+/ST^-$ strains, a statistically significant difference ($p=0.05$, two-tailed Fisher's Exact Test). On the other hand, 10 of 14 $LT^+/ST^-$ strains produced type 1 somatic pili versus none of the nine $LT^+/ST^+$ strains. CFA/I and CFA/II in $LT^-/ST^+$ strains occurred less frequently than in $LT^+/ST^+$ strains and type 1 somatic pili were uncommon.

CFA/I and II were never encountered within the same strain. One strain possessed both CFA/I and type 1 somatic pili.

The E. coli comprised a wide array of serotypes; some were non-typable. The most frequently encountered O serogroups from 19 infections included O159 (6 isolates; Kenya, Korea), O6 (4 isolates; Morocco, Honduras, Zaire), Ox2 (4 isolates; Morocco), O27 (3 isolates; Morocco, Honduras, Kenya) and O20 (2 isolates; Morocco). The remaining 17 infections were spread among a wide array of serogroups or were untypable.

Of the three strains with CFA/I, in two the O group could not be determined because of spontaneous agglutination, while the third was O20. All four CFA/II-positive ETEC from persons with single strains infections were O6 (Table 3), as was the CFA/II-positive strain from one of the persons with multiple infection.

Discussion:

CFA/I and II[17,18] of human ETEC are analogous to K88 and K99 antigens of porcine and bovine ETEC, respectively. All four pilus antigens are similar morphologically when seen in electron photomicrographs; all preferentially appear after cultivation at 37°C on solid agar but are not expressed after culture at 18°C; CFA/I, CFA/II, K88 and K99 cause MRHA of certain erythrocytes.

Veterinary experience with purified K88, K99 and 987 type purified pili vaccines has been so promising[3-6] that it is now believed that up to 90% of ETEC diarrhea in piglets may be prevented by a trivalent pilus antigen vaccine. Since CFA/I and II are considered accessory virulence properties in strains that possess them, there is great expectation that purified CFA/I and II pili may stimulate protective immunity in man against ETEC strains possessing the homologous antigen. However, the ultimate benefit to be derived from a CFA/I, II vaccine, no matter how great the efficacy, will depend on the frequency with which these antigens occur in ETEC strains that cause diarrhea in man. There has been considerable controversy on this point. Evans et al have claimed that CFA/I occurs in 89% of ETEC strains[17], while Gross et al[19] and Orskov and Orskov[20] have identified these antigens in only a small percentage of ETEC from human diarrhea. It could be argued that reports with low prevalence included a preponderance of strains that had lost their CFA plasmids. However, a previous report as well as the current data refute this[8]. We previously demonstrated that many ETEC strains lacking CFA/I and II nevertheless caused diarrhea in volunteers, colonized the intestine and resulted in brisk immune responses.[8] From those observations we concluded that colonization factors distinct from CFA/I and II, or other adhesive factors, must exist in many ETEC that promote attachment to mucosa of the proximal small intestine. Data in the current study support this concept. While CFA/I or II occurred in four of nine $LT^+/ST^+$ strains (44%), such strains represented the causative agent in only one-fourth of the 36 pure ETEC infections. Of the 13 $LT^-/ST^+$ and 14 $LT^+/ST^-$ infections, CFA/I or II was found in only three cases, all $LT^-/ST^+$.

Thus, the common antigens involved in attachment to mucosa must be identified in the remaining majority of strains that lack CFA/I or II; these would have to be included in a future pilus vaccine in order for it to provide broad-spectrum prophylaxis. It is interesting to note that while none of the 14 $LT^+/ST^-$ strains had

CFA/I or II, 10 (71%) had type 1 somatic pili. The antigenic composition of these type 1 somatic pili should be investigated to determine if a few common antigenic types predominate. For example, Brinton[21] reports that 40% of the ETEC strains in his collection produce type 1 somatic pili of an antigenic variety identical to, or closely resembling, those of E. coli H10407. The prevalence of type 1 somatic pili in the $LT^+/ST^+$ and $LT^-/ST^+$ strains in this study was much lower than that reported by Brinton. The reasons for this are unclear but may be related to the fact that in many instances cultures originated from a single clone. Although the precise role of type 1 somatic pili as an adhesive factor mediating attachment of ETEC to small intestinal mucosa is unclear, type 1 somatic pili mediate adhesion of ETEC to epithelial cells in vitro[22-24] and attachment of ETEC can be prevented by specific anti-pilus antibody or competitively inhibited with purified homologous pili.[24] Whatever their function, purified type 1 somatic pili of E. coli H10407 have been inoculated into volunteers as a parenteral vaccine and have proven to be highly immunogenic. Two preliminary volunteer studies have shown some protection against experimental challenge. A multivalent pilus vaccine consisting of two or three prevalent antigenic varieties of type 1 somatic pili, in addition to CFA/I and II, may provide broad-spectrum protection against ETEC diarrhea.

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Table 1

CLINICAL RESPONSE OF VOLUNTEERS FOLLOWING PRIMARY IMMUNIZATION WITH 900 MCG OF PURIFIED H10407 TYPE 1 SOMATIC PILI VACCINE AND FOLLOWING BOOSTER INOCULATIONS WITH 450 OR 180 MCG

| Vaccine Group | N | Primary Immunization Dose (mcg) | Booster Immunization Dose | Adverse Reactions | | |
|---|---|---|---|---|---|---|
| | | | | Fever | Malaise | Local Reactions |
| I | 11 | 900 | 450 | 0 | 0 | 0 |
| II | 10 | 900 | 180 | 0 | 0 | 0 |

Table 2

GEOMETRIC MEAN TITERS AFTER PRIMARY AND BOOSTER IMMUNIZATION OF VOLUNTEERS WITH *E. COLI* TYPE 1 SOMATIC PILI VACCINE

| Primary (Day 0) | Booster (Day +28) | N | Circulating ELISA Pili Antibody Reciprocal Titer $X10^{-3}$ Day: 0 | +28 | +56 |
|---|---|---|---|---|---|
| 900 | 180 | 10 | 2.9 | 94.3 | 111.2 |
| 900 | 450 | 11 | 2.7 | 87.7 | 123 |
| 900(3)* or 1800 (5) | 1800 | 8 | 2.7 | 164.5 | 159 |

*These 8 volunteers were immunized in the 1979 preliminary studies.

Table 3

DISTRIBUTION OF RECIPROCAL TITERS OF ANTI-PILI ANTIBODY ONE MONTH FOLLOWING BOOSTER IMMUNIZATION WITH TYPE 1 SOMATIC PILI VACCINE

| Immunizing Dose | | | Reciprocal Titer of Anti-pili Antibody on Day +56 | | | |
|---|---|---|---|---|---|---|
| Primary (Day 0) | Booster (Day +28) | N | <50 | 51-100 | 101-200 | >200 |
| 900 | 180 | 10 | 20* | 30 | 20 | 20 |
| 900 | 450 | 11 | 9 | 36 | 27 | 27 |
| 900 or 1800 | 1800 | 8 | 25 | 13 | 13 | 50 |

*% of vaccinees with titer in this range

Table 4

RESPONSE OF VACCINEES IMMUNIZED WITH 900 MCG PRIMARY AND 450 MCG BOOSTER DOSES OF PURIFIED *E. COLI* TYPE 1 SOMATIC PILI VACCINE AND CONTROLS FOLLOWING INGESTION OF $5X10^8$ VIRULENT ENTEROTOXIGENIC *E. COLI* (STRAIN H10407)

| Group | Mean Incubation (hr.) | Diarrhea | Mean Total Diarrheal Stool Volume per Ill Volunteer | Mean Total No. Loose Stools per Ill Volunteer | Vomiting | Malaise | Fever | Positiv Stool Culture |
|---|---|---|---|---|---|---|---|---|
| Vaccinees | 31 | 3/6 | 4.31 (0.9-10.6) | 15.3 (5-31) | 2/3 | 2/3 | 1/3 | 6/6 |
| Controls | 36.5 | 7/8 | 2.96 (0.5-8.6) | 12.3 (3-22) | 3/7 | 6/7 | 4/7 | 8/8 |



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Table 5

O ANTIBODY RESPONSE OF PILI VACCINEES AND CONTROLS CHALLENGED WITH E. COLI H10407 (O78:H11) MEASURED BY PASSIVE HEMAGGLUTINATION USING CVD H10407 O ANTIGEN*

| | Day of Challenge Study | | |
|---|---|---|---|
| Volunteer | 0 | +10 | +30 |
| 4002-1 | 2† | ≥512 | 128 |
| " -2 | 32 | 256 | 32 |
| " -3 | 2 | ≥512 | 128 |
| " -4 | 2 | ≥512 | 128 |
| " -5 | 4 | 256 | 32 |
| " -7 | 4 | 64 | 16 |
| " -9 | 2 | ≥512 | 128 |
| " -10 | 8 | ≥512 | 128 |
| " -11 | 4 | ≥512 | ≥512 |
| " -12 | 8 | 256 | 16 |
| " -13 | 8 | ≥512 | 256 |
| " -14 | 8 | ≥512 | 64 |
| " -15 | 16 | 256 | 64 |
| " -16 | <2 | 512 | 64 |
| " -18 | 8 | 128 | 32 |

* heated alkaline LPS extract
† reciprocal titer

Table 6

SERUM O ANTIBODY PRE-AND TEN DAYS POST-IMMUNIZATION WITH H10407 PURIFIED TYPE 1 SOMATIC PILI VACCINE MEASURED BY PASSIVE HEMAGGLUTINATION USING O ANTIGENS FROM E. COLI H10407 (O78) AND E. COLI B7A (O148)

| Volunteer | Primary Immunization Dose (mcg) | Four-fold or Greater Rises in Titer: | | H10407 O Titers | |
|---|---|---|---|---|---|
| | | B7A Antigen | H10407 Antigen | Day 0 | Day +10 |
| 4001-1 | 45 | - | +* | 4 | 32 |
| " -2 | " | - | - | | |
| " -3 | " | - | - | | |
| " -5 | 90 | - | - | | |
| " -6 | " | - | - | | |
| " -7 | " | - | - | | |
| " -8 | " | - | + | 8 | 64 |
| " -9 | 900 | - | + | <2 | 8 |
| " -10 | " | - | - | | |
| " -11 | " | - | + | 2 | 32 |
| " -12 | " | - | - | | |
| " -13 | 1800 | - | + | 2 | 4096 |
| " -14 | " | - | + | 8 | 128 |
| " -15 | " | - | + | <2 | 32 |
| " -16 | " | - | + | 4 | 64 |
| " -17 | " | - | + | 8 | 128 |
| " -18 | " | - | + | 8 | 512 |
| " -19 | " | - | + | <2 | 64 |
| " -20 | " | - | + | <2 | 64 |
| " -21 | " | - | + | <2 | 128 |
| " -23 | " | - | + | 4 | 16,384 |

+* = 4-fold rise

Table 7

SEROCONVERSION RATES FOR O ANTIBODY IN RECIPIENTS OF TYPE 1 SOMATIC PILI VACCINE MEASURED BY PASSIVE HEMAGGLUTINATION USING BUTANOL EXTRACTED (PITTSBURGH) OR HEATED ALKALINE EXTRACTED (CVD) *E. COLI* H10407 O ANTIGEN

| H10407 O Antigen | Pili Vaccine Dose (mcg) | | | | |
|---|---|---|---|---|---|
| | 45 | 90 | 900 | 1800 | Total |
| Pittsburgh (1:300) | 1/3* | 1/4 | 1/4 | 10/10 | 13/21 |
| CVD (1:300) | 1/3 | 0/4 | 2/4 | 10/10 | 13/21 |
| CVD (neat) | 1/3 | 1/4 | 2/4 | 10/10 | 14/21 |

*No. with significant titer rise/No. immunized

Table 8

SERUM O ANTIBODY IN PRE- AND POST-CHALLENGE
SERA OF VOLUNTEERS FED $10^8$ ENTEROTOXIGENIC *E. COLI* B7A (O148:H28)
MEASURED BY PASSIVE HEMAGGLUTINATION USING
B7A (O148) AND H10407 (O78) ANTIGENS

| Volunteer | Serum Specimen | O Antigen Preparations | | |
|---|---|---|---|---|
| | | B7A* | H10407 CVD* | H10407 Pittsburgh† |
| 2005-5 | Day 0 | 128** | 8 | 64 |
| | Day +10 | 64 | 16 | 128 |
| 2005-6 | Day 0 | 2 | 0 | 8 |
| | Day +10 | 64 | 0 | 8 |
| 2005-12 | Day 0 | 32 | 0 | 0 |
| | Day +10 | 256 | 0 | 0 |
| 2005-13 | Day 0 | 8 | 4 | 16 |
| | Day +10 | 128 | 4 | 16 |
| 2005-17 | Day 0 | 16 | 2 | 16 |
| | Day +10 | 128 | 2 | 16 |

* heated NaOH extraction
† butanol extraction
** reciprocal titer

Table 9

IgM ELISA ANTIBODY RISES TO H10407 O ANTIGENS IN PRE- AND TEN DAY POST-CHALLENGE SERA OF VOLUNTEERS FED *E. COLI* H10407

| | O Antigen Preparation | |
|---|---|---|
| Volunteer | Pittsburgh | CVD |
| 4002-1 | +* | + |
| " -2 | - | - |
| " -3 | + | - |
| " -4 | + | + |
| " -5 | + | + |
| " -9 | + | + |
| "-10 | + | + |
| "-11 | + | + |
| "-12 | - | + |
| "-13 | + | + |
| "-14 | + | + |
| "-16 | + | + |
| "-18 | - | - |
| Total | 10/13 | 10/13 |

+ = significant rise in ELISA titer on day +10 over day 0

Table 10

IgM ELISA ANTIBODY RISES TO H10407 (O78) ANTIGENS IN PRE- AND 10 DAY POST-CHALLENGE SERA OF VOLUNTEERS INFECTED WITH E. COLI B7A (O148)

| | H10407 O Antigen Preparation | |
|---|---|---|
| Volunteer | Pittsburgh | CVD |
| 2005-5 | -* | - |
| "-12 | - | - |
| "-15 | - | - |
| "-18 | - | - |
| " -6 | - | - |
| "-13 | - | - |
| "-16 | - | - |
| "-20 | - | - |
| "-11 | - | - |
| "-14 | - | - |
| "-17 | - | - |
| "-21 | - | - |

* - = no significant rise in ELISA antibody

Table 11

IgM ELISA ANTIBODY PRE-AND TEN DAYS POST-
IMMUNIZATION WITH TYPE 1 SOMATIC PILI VACCINE

| Volunteer | | H10407 O Antigen Preparation | |
|---|---|---|---|
| | | Pittsburgh | CVD |
| 4001-1 | 45 | - | - |
| " -2 | " | - | - |
| " -3 | " | - | - |
| " -5 | 90 | - | - |
| " -6 | " | - | - |
| " -7 | | - | - |
| " -8 | 900 | - | - |
| " -9 | " | - | - |
| "-10 | " | - | - |
| "-11 | " | - | - |
| "-12 | 1800 | - | - |
| "-13 | " | +* | + |
| "-14 | " | - | + |
| "-15 | " | - | - |
| "-16 | " | - | - |
| "-17 | " | - | - |
| "-18 | " | + | + |
| "-20 | " | - | + |
| "-21 | " | + | + |
| "-22 | " | + | + |

+* = significant rise in antibody

Table 12

PREVALENCE OF CFA/I, CFA/II AND TYPE 1 SOMATIC PILI IN ENTEROTOXIGENIC *ESCHERICHIA COLI* ISOLATED FROM PATIENTS† WITH ACUTE TRAVELERS' DIARRHEA: RELATIONSHIP WITH TOXIN TYPE

| Enterotoxin Phenotype | Class of Pili | | |
|---|---|---|---|
| | CFA/I | CFA/II | Type 1 Somatic |
| $LT^+/ST^+$ | 2/9* (22%) | 2/9 (33%) | 1/9 |
| $LT^-/ST^+$ | 1/13 (15%) | 2/13 (15%) | 4/13 (31%) |
| $LT^+/ST^-$ | 0/14 | 0/14 | 10/14 (71%) |
| Totals | 4/36 (11%) | 5/36 (14%) | 14/36 (39%) |

† Patients with infections from which enterotoxigenic *E. coli* (ETEC) of only a single serotype and toxin type were isolated.

* Infections due to ETEC with pilus/no. infections.

FIG 1

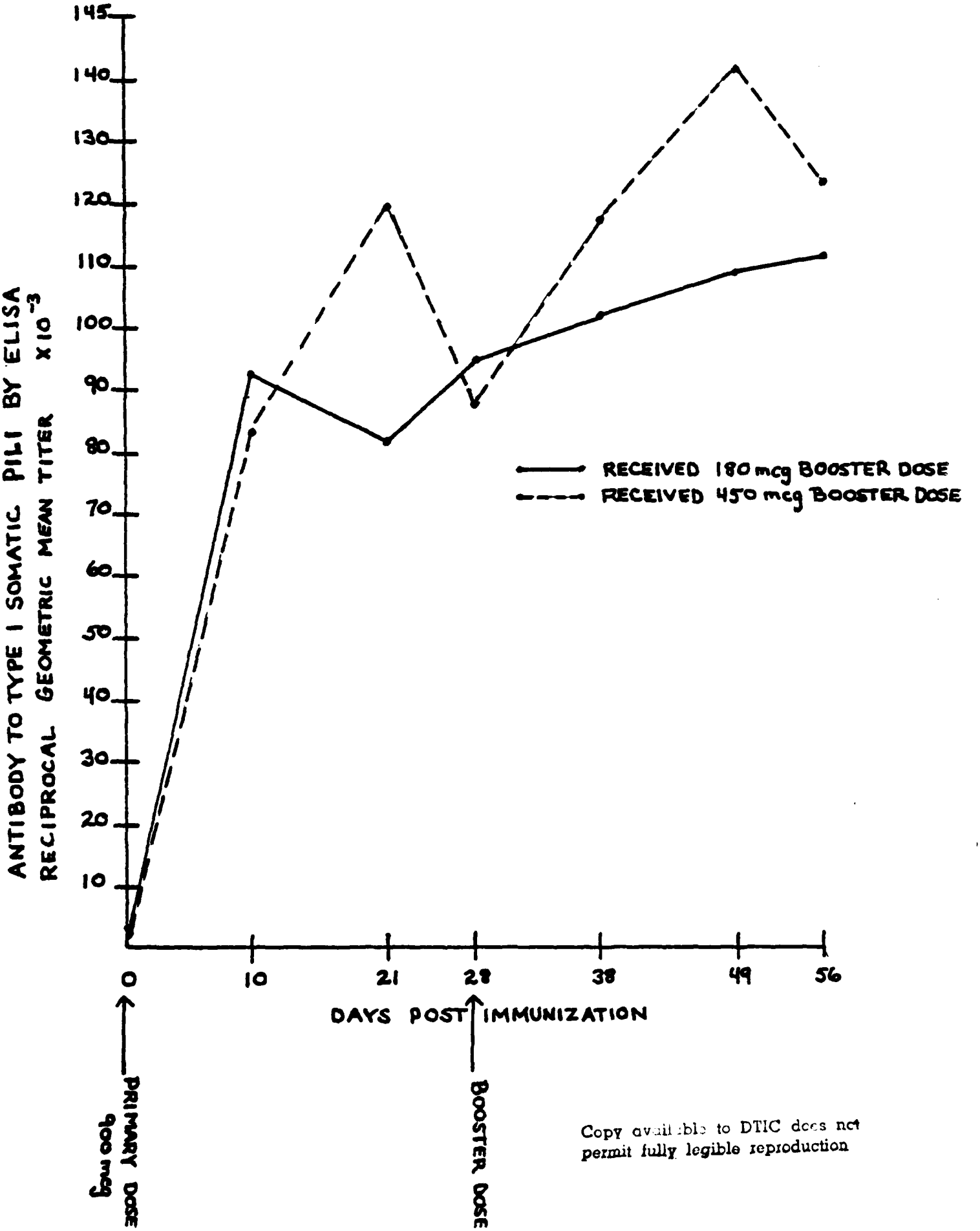


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FIG 2

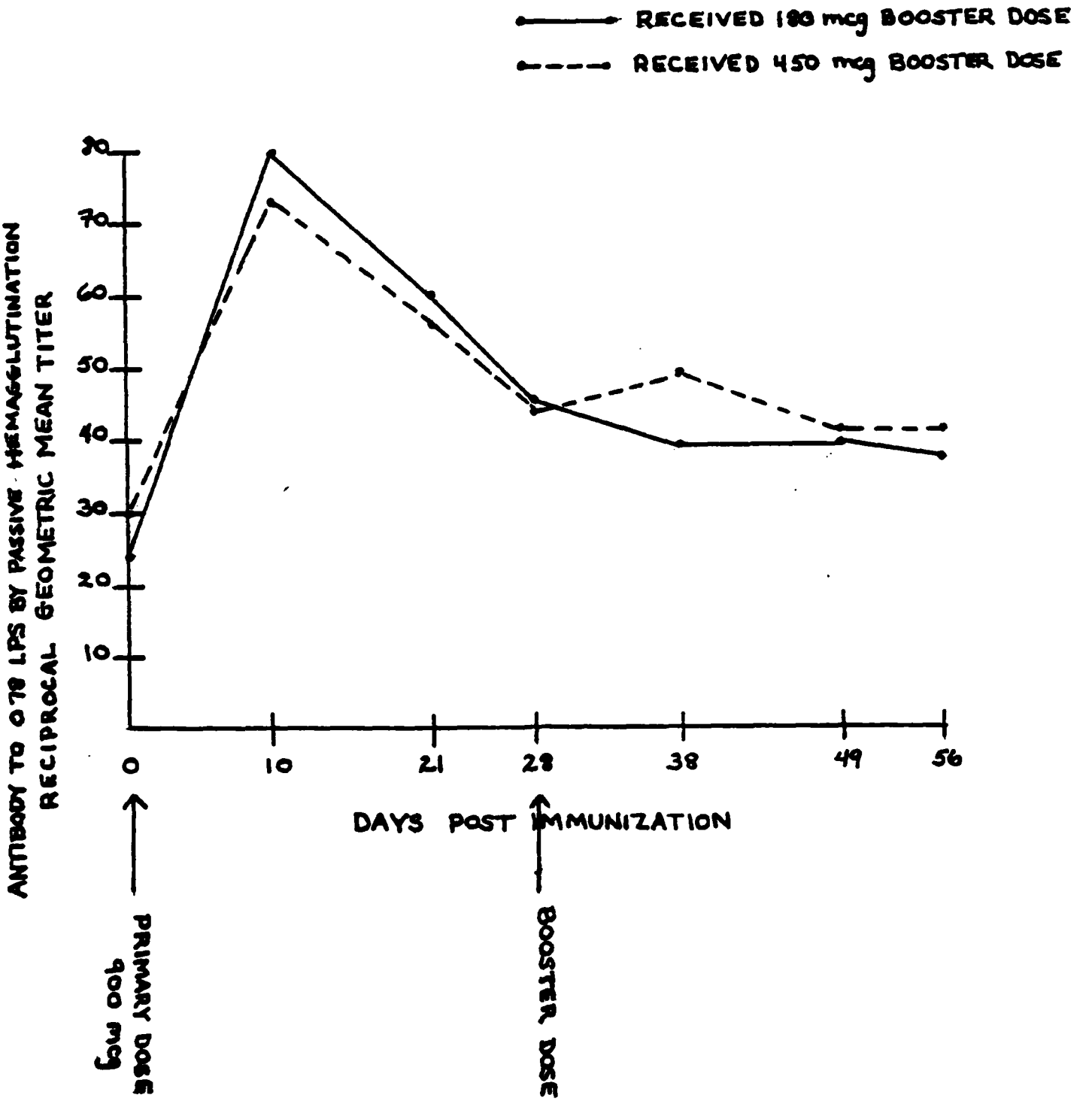

RECEIVED 180 mcg BOOSTER DOSE
RECEIVED 450 mcg BOOSTER DOSE
ANTIBODY TO 078 LPS BY PASSIVE HEMAGGLUTINATION
RECIPROCAL GEOMETRIC MEAN TITER
80
70
60
50
40
30
20
10
0
10
21
28
38
49
56
DAYS POST IMMUNIZATION
PRIMARY DOSE
100 mcg
BOOSTER DOSE

FIG 3

O ANTIBODY MEASURED BY PASSIVE HEMAGGLUTINATION IN RECIPIENTS OF PILI VACCINE : CORRELATION OF ANTIGEN AT NEAT AND 1:300 DILUTION

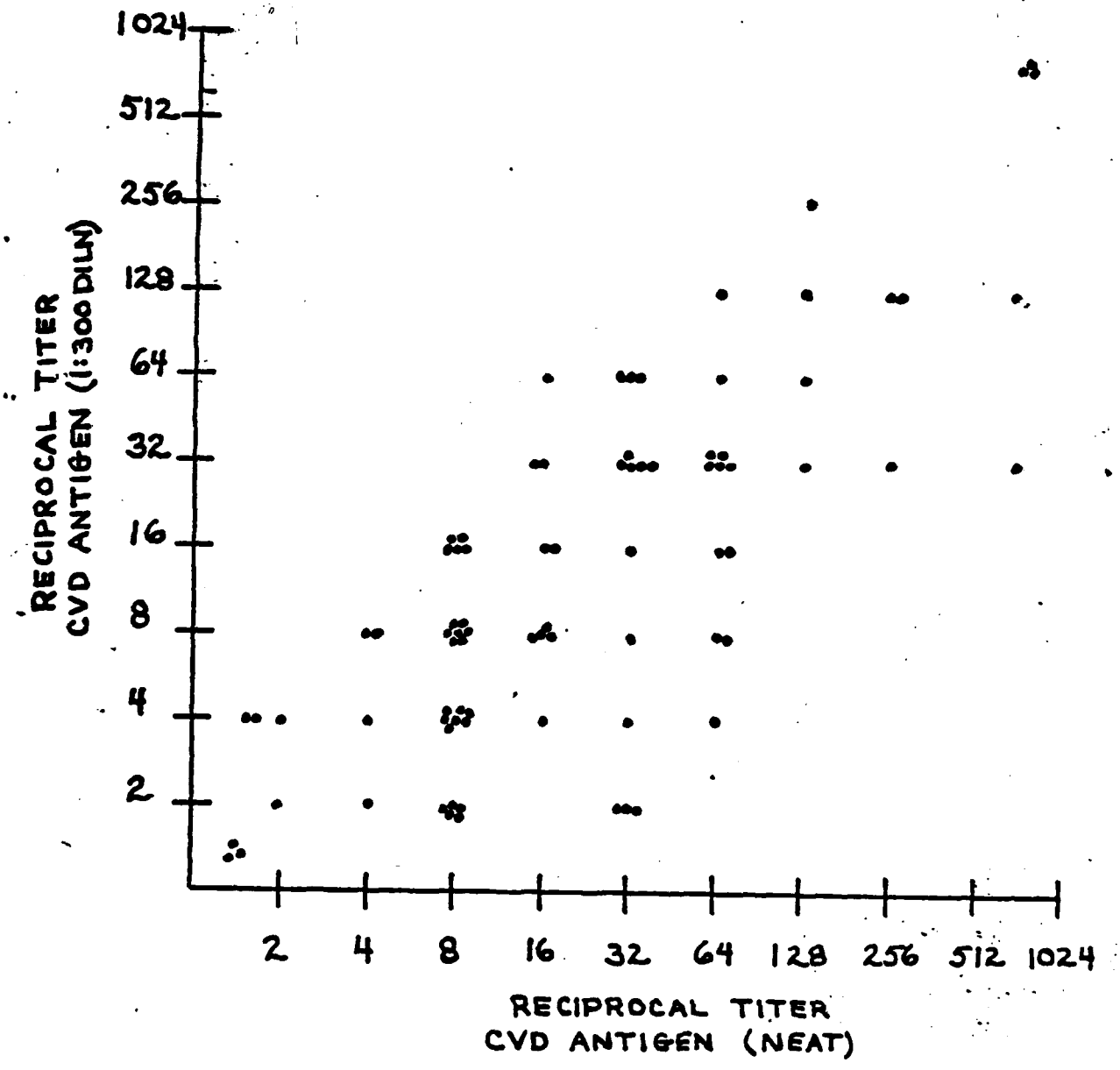


FIG 4

O ANTIBODY IN RECIPIENTS OF PILI VACCINE MEASURED BY PASSIVE HEMAGGLUTINATION : COMPARISON OF BUTANOL EXTRACTED (PITTSBURGH) AND HEAT/ALKALI EXTRACTED (CVD) LIPOPOLYSACCHARIDE ANTIGENS

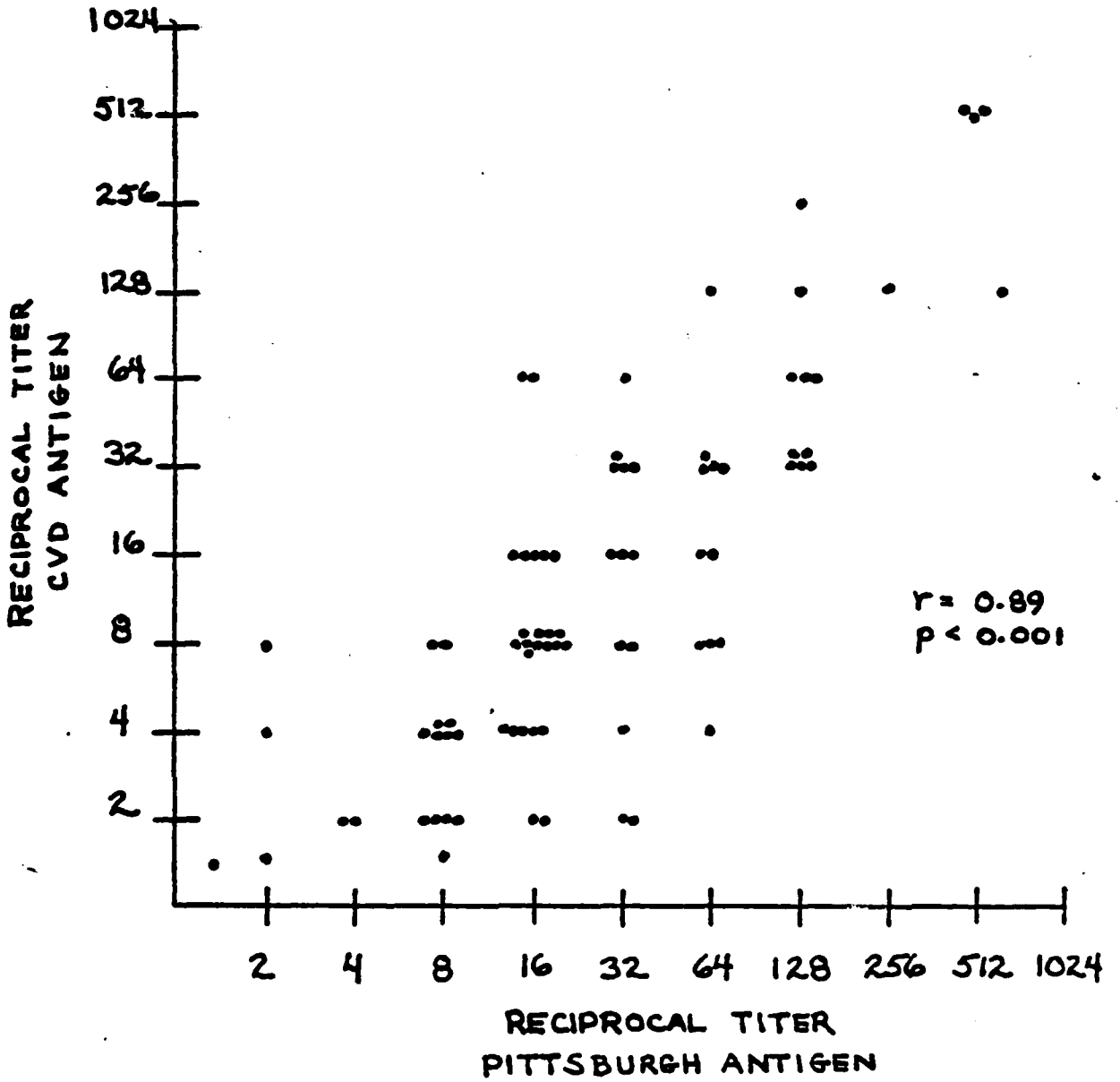
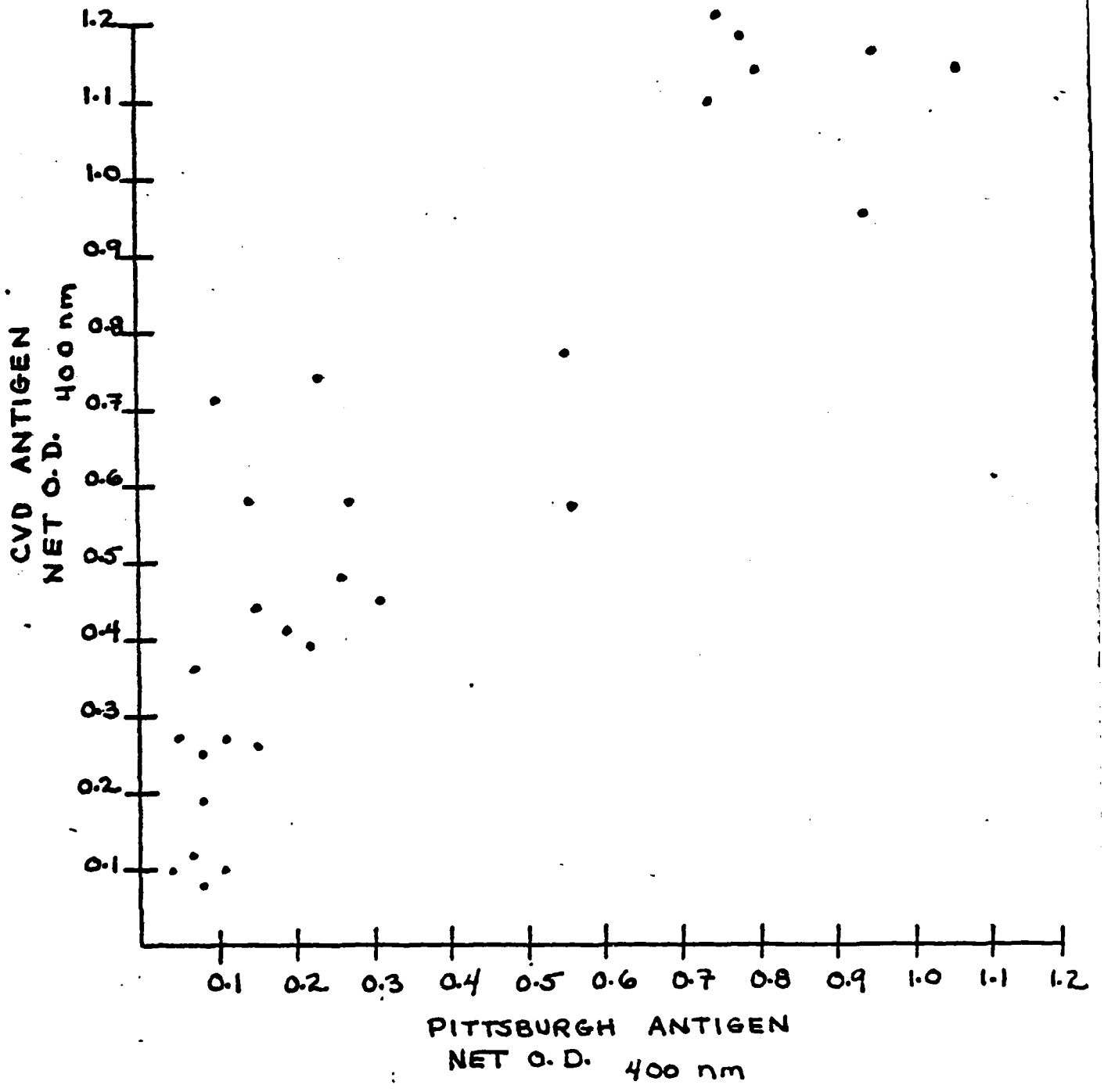


FIG 5

CORRELATION OF IgM ANTIBODIES MEASURED BY ELISA TO E COLI O 78 ANTIGENS PREPARED BY TWO METHODS. SERA FROM IMMUNIZED AND UNIMMUNIZED VOLUNTEERS CHALLENGED WITH VIRULENT E COLI H10407.



## PUBLICATIONS OF CONTRACTED-SUPPORTED WORK

### 1980 CONTRACT YEAR

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Appendix A

# REACTOGENICITY AND EFFICACY STUDIES OF ESCHERICHIA COLI H10407 PURIFIED TYPE 1 SOMATIC PILI VACCINE

Myron M. Levine, M.D., D.T.P.H., Charles C. Brinton, Jr., Ph.D., Alfred Saah, M.D. Sylvia O'Donnell, R.N., Peter Fusco, Ph.D., Timothy P. Hughes, B.S., Roy Robins-Browne, M.B., B.Ch., Malvi Solomon, M.D., Margaret B. Rennels, M.D., Charles R. Young, B.A., Sarah Wood, Ph.D.

Center for Vaccine Development,
Division of Infectious Diseases,
University of Maryland School of Medicine
Baltimore, Maryland 21201

Laboratory for Pilus Research and Development,
Department of Biological Sciences,
University of Pittsburgh,
Pittsburgh, Pennsylvania 15260

ABSTRACT

Primary immunization with purified type 1 somatic pili vaccine prepared from enterotoxigenic Escherichia coli (ETEC) H10407 was given intramuscularly to 21 adult volunteers in doses of 45 (3), 90 (4), 900 (4), or 1800 (10) mcg. without adverse reactions. Vaccine did not significantly alter intestinal transit time, D-xylose absorption or the prevalence of normal colonic flora E. coli that possess type 1 somatic pili of the H10407 antigenic variety. Local reactions, characterized by heat, induration, erythema or tenderness (which lasted less than 48 hrs. in all but one instance), were seen in six of 15 vaccinees who received an 1800 mcg booster inoculation one month after primary.

One month after booster inoculation, six vaccinees who received two doses of purified pili and 7 unimmunized control volunteers were challenged with $5X10^8$ ETEC H10407 organisms. Diarrhea occurred in all 7 controls, including copious, cholera-like purging in three persons, of whom two required intravenous rehydration. Vomiting occurred in 6 of 7 controls, malaise in seven and fever in two. Only 2 of 6 vaccinees developed diarrhea ($p=0.04$) and neither manifested malaise, vomiting or fever. Mean D-xylose blood absorption values were significantly diminished in both vaccinees and controls post-challenge in comparison with pre-challenge. Despite clinical protection, all vaccinees as well as controls excreted E. coli H10407. Of 602 H10407 clones from coprocultures examined during challenge, 100% possessed both type 1 somatic and NMS pili.

In preliminary studies, the E. coli H10407 purified type 1 somatic pili parenteral vaccine has been shown to be non-reactogenic after a single dose, to cause mild local reactions in 1/3 of subjects following booster inoculation and to provide significant clinical protection in the face of a strenuous challenge test. These results encourage further studies and confirm the feasibility of immunologic control of ETEC diarrhea in man.

## INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC), the most frequent etiologic agent of travelers' diarrhea, usually account for 30-60% of cases.[1,2] Thus, an effective vaccine against ETEC could greatly diminish the risk of enteric illness in travelers. It has been shown in recent years that various types of pili (hair-like organelles on the bacterial cell surface) serve as virulence factors for ETEC of man and animals.[3-9] Vaccines consisting of purified K88,[10] K99[11,12] and 987-type[13] pili have been found to be safe and highly protective in preventing severe ETEC diarrhea in piglets and calves. Piglets or calves suckled on immunized mothers were protected against otherwise lethal challenge with E. coli bearing the homologous pilus antigen.[10-13]

If only a few antigenic types of colonization factors were found to be common to human ETEC and if they could be purified for use as vaccines, immunoprophylaxis of human ETEC disease would be feasible.[5,14] Among the components of a polyvalent pilus vaccine might be the so-called colonization factor antigen pili I and II (CFA/I, CFA/II)[8,9] associated with non-mannose-sensitive (NMS) hemagglutination of certain erythrocytes. Other antigens would have to be included to protect against the large number of ETEC pathogens lacking CFA/I or II.[14-16] Type 1 somatic pili may serve this role. They manifest adhesive properties for epithelial cells,[17-19] exhibit mannose-sensitive hemagglutination[20,21] and are common in ETEC pathogens.[5,14] According to Brinton et al,[5] approximately 40% of the ETEC that elaborate type 1 somatic pili possess an antigenic variety identical or immunologically closely related to that of E. coli H10407. This observation suggests that E. coli H10407 type 1 somatic pili could serve as an important component in a multiple pilus antigen vaccine against human diarrhea.

In this report we describe the reactogenicity and efficacy in adult volunteers of a parenteral E. coli H10407 purified type 1 somatic pilus vaccine.

## MATERIALS AND METHODS

### Vaccine

E. coli H10407 (O78:H11) elaborates heat-labile (LT) and heat-stable enterotoxins and possesses two types of non-sex pili: type 1 somatic pili[5,22] (associated with mannose-sensitive hemagglutination of guinea pig erythrocytes) and NMS pili[5] (associated with non-mannose-sensitive hemagglutination of human erythrocytes). NMS pili are probably identical to CFA/I.[8,23]

Type 1 somatic pili from cloned E. coli H10407 were purified as described elsewhere.[24,25] Vaccine was contained in 50 ml vials in a concentration of 1800 mcg of purified pili protein per ml. Merthiolate served as preservative.

### Volunteers

Volunteers were college students and other healthy adults (mean age 24 years). Reactogenicity and vaccine efficacy challenge studies were carried out in the 22 bed Isolation Ward of the Center for Vaccine Development. The protocol was approved by the University of Maryland Hospital Human Volunteer Research Committee and the Clinical Review Sub-Panel of the National Institute of Allergy and Infectious Diseases. The studies were explained to volunteers in detail and signed, witnessed consent was obtained. The informed nature of consent was documented prior to inoculation by requiring all volunteers to pass a written examination containing multiple choice and true-false questions on all aspects of the study, including purpose, hazards, procedures and pertinent bacteriology and immunology.[26] The pre-inoculation health status of volunteers was ascertained from medical history, physical examination, chest radiograph, electrocardiogram, complete blood count, negative pregnancy test, urinalysis, blood chemistries (including serum glucose, urea nitrogen and electrolytes), and tests for liver function, syphilis and hepatitis B surface antigen.

Immunization

Sequential groups of 3-6 volunteers (21 in total) were parenterally inoculated in the triceps muscle with 45, 90, 900 or 1800 mcg doses of purified pili vaccine. Twenty-eight days thereafter 15 of the 21 volunteers received a booster IM inoculation with 1800 mcg of pili vaccine. Volunteers who received 1800 mcg primary or booster inoculations were also given an inoculation with the same volume of physiological saline in the other arm, in order to assess local reactions due to pili rather than the act of IM inoculation per se. Neither the volunteer nor the examining physician was told which arm received vaccine and which received saline.

Challenge Study:

Approximately one month following inoculation with the 1800 mcg booster dose (two months after primary immunization), six vaccinees and seven un-immunized control volunteers participated in a challenge study to assess vaccine efficacy.

Cloning of the Challenge Strain:

The E. coli H10407 parent strain utilized in previous challenge studies[27] was inoculated into Z broth, incubated for 4 hrs. with shaking at 37°C and frozen at -70°C with DMSO. The frozen stock was later thawed and streaked onto casamino-yeast extract (CAYE) agar plates. Two colonial types were isolated and tested for hemagglutination pattern during several passages. The smaller, less-dense colony, designated H10407/ML/CB/C1PF-003, demonstrated non-mannose sensitive hemagglutination of human erythrocytes and weak mannose-sensitive hemagglutination of guinea pig erythrocytes. Using agglutination with specific anti-pili antisera and immuno-labeling with electron microscopy, it was confirmed that this clone 003 possessed both type 1 somatic and NMS pili.

Inocula and Challenge:

E. coli strain H10407/ML/CB/C1PF-003, which elaborates both heat-labile (LT) and heat-stable (ST) enterotoxins, was thawed from storage in skim milk at -70°C and streaked onto CAYE agar. After 15 hrs. incubation at 37°C 16 piliated colonies identified under stereo-microscope were picked and streaked on CAYE agar. Twelve hrs. after incubation at 37°C, 30 piliated colonies were used to heavily inoculate each of six CAYE agar plates for incubation at 37°C. After 12 hrs., the CAYE agar cultures were harvested with saline (0.85%) and dilutions made in saline.

Two gm. of $NaHCO_3$ were dissolved in 150 ml of distilled water of which the volunteers drank 120 ml; one minute later they drank the remaining 30 ml. in which the E. coli inoculum ($5X10^8$ organisms) was suspended. Inoculum size was quantitated by replicate pour-plate technique before and after challenge. The presence of both type 1 somatic and NMS piliation on the challenge organisms was documented by agglutination with specific antisera.

Clinical Observations:

Immunization:

Volunteers were kept under close observation on the Isolation Ward for two days post-inoculation with pili vaccine. Oral temperatures were taken every six hrs. and injection sites were examined for erythema, heat, induration and tenderness.

Challenge:

Volunteers were examined daily starting three days prior to ingesting the virulent organisms. Oral temperatures were taken every six hrs. and repeated within five minutes if they were 37°C or above. All stools and vomitus were collected in plastic cholera seats, examined by a nurse or physician and volumes measured. Stools were graded on a five point scale[28] - grades 1 (fully

formed) and 2 (soft) were considered normal; grade 3 denoted thick liquid stool, grade 4 opaque-watery, and grade 5 rice-water stools. Diarrhea was defined as three or more loose (grade 3-5) stools in 24 hrs. or at least two loose stools within 24 hrs. surpassing 250 ml. in volume. Prior to discharge all volunteers received a five day course of oral neomycin (500 mg six hourly) to eradicate fecal carriage of the virulent ETEC strain.[27]

Tests of Gastrointestinal Function:

Prior to primary immunization, prior to booster inoculation and before and after challenge with virulent ETEC, intestinal absorption and transit time were measured in most of the volunteers.

Intestinal transit time was determined by Carmine red dye technique.[29] A 500 mg oral dose of dye was ingested by the volunteers and the stools examined for evidence of the dye.

Intestinal absorption was assessed by D-xylose absorption[30,31] and excretion tests.[32] After an overnight fast, volunteers micturated, provided a baseline blood specimen and ingested 5.0 gm of D-xylose in 250 ml of distilled water. An additional 250 ml of water was then consumed. Blood for measurement of D-xylose was collected after one hour.[30,31] For D-xylose excretion tests urine was collected for five hours after ingestion of the monosaccharide.[32] D-xylose was quantitated by the method of Roe and Rice.[33]

Stool Cultures:

During Immunization:

Stool specimens from 15 vaccinees were collected before and 28 days after primary immunization and plated on Levine's eosin-methylene-blue (EMB) agar. Fifteen clones per specimen were inoculated into separate tube of Mueller-Hinton broth (5 ml) and incubated resting for 48 hrs. at 37°C. Sub-cultures were made into Mueller-Hinton broth and similarly cultured. The culture tubes

were centrifuged, the supernatants discarded and the bacterial pellets tested for agglutination with antiserum to purified H10407 type 1 somatic pili; the proportion of cultures reacting with specific antibody were recorded.

During Challenge:

Stool specimens or rectal swabs were collected daily throughout the challenge study (including at least two pre-challenge specimens) and inoculated onto Levine's EMB agar. Ten colonies with a typical E. coli metallic sheen were sub-cultured onto slants of trypticase soy agar in screw-top tubes. After 18 hrs. of incubation at 37°C these clones were tested for agglutination with rabbit antisera to E. coli H10407, H10407 type 1 somatic pili and H10407 NMS pili. The isolates were also tested for LT production by Y-1 adrenal cell assay as previously described.[34,35]

RESULTS:

Reactogenicity:

Clinical:

Neither erythema, induration, heat, tenderness, fever nor malaise occurred in any of the 21 volunteers who received primary immunization with varying dose of purified pili vaccine (Table 1). Among the fifteen persons who received an 1800 mcg booster inoculation no systemic adverse reactions were noted but six vaccinees developed objective local adverse reactions including induration, heat or erythema (Table 2). Local reaction following the booster occurred in persons who had received primary inoculations with 45 (2), 900 (2) and 1800 (2) mcg doses of vaccine. Local reactions were evident 24 hrs post-inoculation but, with one exception, were gone by 48 hrs. The reactions were described as mild to moderate by the volunteers. In no instance did nausea, vomiting, diarrhea or fever occur.

Gastrointestinal Function:

Approximately two-thirds of the vaccinees had measurements of intestinal

transit time and D-xylose absorption before and 28 days following primary immunization. In no instance were abnormal values noted. The mean intestinal transit times pre-immunization (mean 29 hrs., range 12-72 hrs.) were similar to those post-immunization (mean 20 hrs., range 4-72 hrs.) in the 14 persons tested.

To establish the mean and range of normal values for the one hour blood xylose absorption test in a population of normal adults tested by our laboratory, specimens from 30 healthy adults were run. These 30 individuals comprised 21 pili vaccinees prior to immunization, seven unimmunized control volunteers prior to challenge and two other healthy adults. The mean blood D-xylose level one hour post-ingestion of the monosaccharide was 14.2±5.2 mg/dl (mean ± S.D.).

Sixteen recipients of pili vaccine (Table 3) had D-xylose absorption tests performed prior to and 28 days after primary immunization; the levels before (mean 12.6±4.5 mg/dl) and 28 days after vaccination (mean 14.7±4.1 mg/dl) were similar ($p>0.1$, paired Student's t test).

Six vaccinees who received 1800 mcg booster doses of pili vaccine went on to participate in the challenge study. These individuals provided an opportunity to analyse D-xylose absorption at four points in time (Table 3): pre-immunization (Day 0), pre-booster (day +28), pre-challenge (day +57) and post-challenge (day +64). There were no significant differences in this group in mean one hour blood xylose levels on day 0 (14.8±2.7 mg/dl), day +28 (14.4± 4.0 mg/dl), and day +57 (15.7±2.8 mg/dl) (Table 3).

Colonic E. coli

Stool specimens from 16 vaccinees were cultured prior to and 28 days after primary immunization. The prevalence of normal E. coli flora that possess type 1 somatic pili of the H10407 antigenic variety were recorded.

As seen in Table 4, in 12 of 15 vaccinees prior to immunization, 40-100% of normal E. coli colonic flora were agglutinated by antiserum to H10407 type 1 somatic pili. In ten persons the prevalence of piliated E. coli remained unchanged. In two persons E. coli possessing type 1 somatic pili of H10407 antigenic variety disappeared 28 days post-immunization, while piliated E. coli appeared on day 28 in three persons in whom they were absent pre-immunization. These data demonstrate that vaccine did not alter the prevalence of normal E. coli colonic flora bearing type 1 somatic pili of H10407 antigenic variety.

Vaccine Efficacy:

Clinical:

One month after IM inoculation with an 1800 mcg booster dose of pili vaccine, six vaccinees agreed to participate in a challenge study along with seven unimmunized control volunteers. Following ingestion of $5X10^8$ virulent E. coli H10407 bacteria all seven controls developed diarrheal illness (Table 5). Three controls passed copious rice-water stools resulting in cholera-like total diarrheal stool volumes of 3.8, 7.5 and 9.9 liters; two controls required intravenous fluids to maintain hydration. In contrast, only 2 of 6 vaccinees developed diarrheal illness ($p=0.04$, Fisher's Exact Test). While ill controls experienced malaise (7 of 7) and vomiting (6 of 7), none of the vaccinees, ill or well, had these complaints (Table 5). Otherwise, the diarrheal illness manifested by the two vaccinees was similar in incubation, total volume, number of loose stools and duration to that seen in the controls.

Excretion of E. coli H10407:

Despite clinical protection, all vaccinees, as well as controls excreted virulent E. coli H10407. Within 48 hrs. post-challenge, all volunteers, vaccinees and controls, were shedding E. coli as the predominant aerobic coliform. Of 1200 E. coli clones picked from stool culture plates during the first five days

post-challenge, 1183 (98.6%) were strongly agglutinated by lapine H10407 antiserum. Six hundred and two clones were tested for the presence of H10407 type 1 somatic and NMS pili by agglutination with specific antisera; all 602 clones tested had both types of pili.

D-xylose Absorption Tests

The one hour blood xylose levels fell significantly ($p \leq 0.05$, Table 3) in the seven control volunteers post-challenge in comparison with pre-challenge levels. The mean one hour blood D-xylose level was also significantly lower in the group of six vaccinees in comparison with pre-challenge and pre-immunization levels ($p \leq 0.01$, Table 3). The two vaccinees with diarrhea had the most prominent falls in blood xylose levels between pre- and post-challenge specimens; 16.7 mg/dl fell to 2.1 and 13.6 dropped to 2.8 mg/dl.

Five hour urine collections in vaccinees and controls pre- and post-challenge permitted D-xylose excretion tests to be performed. Pre-challenge all 13 volunteers had normal values ($\geq 1.2$ gm/5 hr. urine volume). Post-challenge six of seven ill controls, both ill vaccinees and one of four well vaccinees had abnormal test results.

DISCUSSION:

The encouraging results in prevention of severe diarrhea due to ETEC in piglets and calves by means of purified pili vaccines[10-13] prompted the current studies in man. Type 1 somatic pili adhere to epithelial cells[17-19] allowing a bacteria/host-cell interaction and approximately 40% of human ETEC surveyed possess type 1 pili of an antigenic variety closely related to those of *E. coli* H10407.[5] Therefore, we elected to evaluate purified H10407 type 1 somatic pili as a parenteral vaccine in man. Single intramuscular doses of 45, 90, 900 and 1800 mcg caused no local or systemic reactions. However, 6 of 15 persons who received an 1800 mcg booster inoculation developed local

adverse reactions lasting approximately 24 hrs. The local reactions were presumably due to antigen-antibody reactions since they occurred only after the booster inoculation. It is not clear whether the reactions were mediated by anti-pili antibody or antibody to the small amount of H10407 endotoxin (O antigen) also present in the vaccine preparation.[25] We expect that further studies involving modification of primary and booster doses of vaccine will lead to an immunogenic, non-reactogenic immunization schedule.

Although the vaccine stimulated high levels of circulating antibody[25] it did not adversely affect gastrointestinal function: intestinal transit time, D-xylose absorption tests (Table 3) and prevalence of normal E. coli colonic flora possessing type 1 somatic pili of H10407 antigenic variety (Table 4) were not significantly altered.

Despite the small numbers of volunteers involved in the challenge study, the difference in attack rates between the control (7/7) and immunized (2/6) groups was significant ($p=0.04$). Protection occurred despite a large inoculum that caused severe disease in the control group. Furthermore, illness in the two vaccinees appeared qualitatively milder since no vomiting, or malaise occurred, while they were the rule in the ill controls. All challenged vaccinees, including those without illness, excreted virulent ETEC H10407. This is not surprising since in homologous re-challenge studies in volunteers with E. coli H10407[27] and B7A[35] we observed that fecal excretion of virulent ETEC was not diminished despite clinical protection. Consequent to the homologous re-challenge studies we hypothesized that protection is mediated by a mechanism that is not bactericidal and probably involves antibody at the mucosal surface that interferes with adhesion of bacteria to critical mucosal sites.[35] We assume that vaccine-derived protective antibody made its way by transudation from the serum to the mucosal surface. It is also possible, although we believe less likely, that parenteral immunization stimulated secretory antipili anti-

body production by the intestine. There exist other examples wherein parenteral vaccines exert notable effects in the intestine. Parenteral killed whole cell cholera vaccines provide protection,[36] albeit short-lived, against Vibrio cholerae, a pathogen which like ETEC does not invade intestinal mucosa. Furthermore, parenteral killed poliovirus vaccine exerts an intestinal effect manifested by diminished intestinal excretion of poliovirus following natural infection or subsequent ingestion of attenuated strains.[37-39]

As described in the accompanying paper,[25] the pili vaccine induced high levels of anti-pili antibody. However, small amounts of contaminating endotoxin (O antigen) present in the vaccine also led to stimulation of anti-O antibody.[25] The O antibody, which is predominantly IgM, fell to low titer by the time of challenge, while the IgG class pili antibody remained high.[25] Thus it is unlikely that antibodies to O antigen rather than pili mediated protection but based on the current data this cannot be ruled out. This dilemma was previously encountered in veterinary studies where the purified pili vaccines also contained small amounts of contaminating O antigen.[12,13,40] To resolve this question we plan to carry out a study in which volunteers immunized with purified H10407 type 1 somatic pili will be challenged with E. coli B7A, and ETEC strain of a different serotype (O148:H28) than H10407 (O78:H11) that possesses type 1 somatic pili antigenically identical to those of H10407. Subsequent studies will also include administration of pili by the oral route in an effort to more effectively stimulate immunity at the mucosal surface.

The results described in this report represent a tangible first step toward immunologic control of ETEC diarrhea. Identification and purification of a few antigenic types of adhesion pili common to most ETEC could lead to an effective polyvalent pilus vaccine if the other antigens prove as protective as the type 1 somatic pili preparation.

# CLINICAL RESPONSE OF VOLUNTEERS TO PARENTERAL IMMUNIZATION WITH PURIFIED H10407 TYPE 1 SOMATIC PILI VACCINE

| Dose | Following Inital Vaccine Dose | | | Following 1800 mcg. Booster Dose | | |
|---|---|---|---|---|---|---|
| | Fever | Malaise | Local Reactions | Fever | Malaise | Local Reactions |
| 45 mcgs. | 0/3* | 0/3 | 0/3 | 0/3 | 0/3 | 2/3 |
| 90 mcgs. | 0/4 | 0/4 | 0/4 | 0/3 | 0/3 | 0/3 |
| 900 mcgs. | 0/4 | 0/4 | 0/4 | 0/3 | 0/3 | 2/3 |
| 1800 mcgs. | 0/10 | 0/10 | 0/10 | 0/6 | 0/6 | 2/6 |

*No. with reactions / No. immunized

TABLE 2

Appendix A

DESCRIPTION AND DURATION OF LOCAL REACTIONS

FOLLOWING THE BOOSTER 1800 MCG. PARENTERAL DOSE OF H10407

TYPE SOMATIC PILI VACCINE

| VOLUNTEER | TENDERNESS | ERYTHEMA | INDURATION | REACTION 24 HRS. | PRESENT AT 48 HRS. |
|---|---|---|---|---|---|
| 4001D-16 | 2+ | 0 | 0 | + | 0 |
| 4001D-18 | 2+ | 80 mm. | 80 mm. | + | 0 |
| 4001C-10 | +/- | 70 mm. | 70 mm. | + | + |
| 4001C-12 | +/- | 0 | 40 mm. | + | 0 |
| 4001A-3 | 1+ | 50 mm. | 0 | + | 0 |
| 4001A-2 | 2+ | 150 mm. | 150 mm. | + | 0 |

Table 3

ONE HOUR D-XYLOSE BLOOD ABSORPTION TESTS IN RELATION TO IMMUNIZATION WITH TYPE 1 SOMATIC PILI VACCINE AND CHALLENGE WITH ENTEROTOXIGENIC ESCHERICHIA COLI

| Group | Day*: 0 | +28 | +57 | +65 |
|---|---|---|---|---|
| 30 Normal Adults | 14.2±5.2† | - | - | |
| 16 Vaccinees | 12.6±4.5 | 14.7±4.1 | - | |
| 6 Vaccinees** | 14.8±2.7 | 14.4±4.0 | 15.7±2.8 | 7.7±4.7†† |
| 7 Controls | - | - | 16.9±7.7 | 9.3±4.2†* |

*Day 0 = pre-immunization
Day 28 = one month after primary immunization
Day 57 = one month after booster immunization and three days pre-challenge with ETEC
Day 65 = five days post-challenge with ETEC.
†mean ± standard deviation mg/dl.
**six vaccinees who participated in vaccine efficacy challenge study.
††Day 65 value significantly lower than day 0 and day +57 ($p<0.01$, Student's paired t test.
†*Day 65 value significantly lower than day pre-challenge ($p<0.05$, Student's paired t test).

TABLE 4

PREVALENCE OF NORMAL COLONIC *E. COLI* FLORA THAT POSSESS SOMATIC PILI OF H10407 ANTIGENIC VARIETY

| Volumteer | Day 0 | Day +28 |
|---|---|---|
| 4001-1A | 0* | 100 |
| 4001-2A | 0 | 100 |
| 4001-3A | 0 | 80 |
| 4001-6B | 93 | 87 |
| 4001-7B | 67 | 100 |
| 4001-8B | 93 | 100 |
| 4001-10C | 100 | 100 |
| 4001-11C | 100 | 100 |
| 4001-12C | 100 | 100 |
| 4001-13D | 40 | 73 |
| 4001-14D | 100 | 0 |
| 4001-15D | 73 | 0 |
| 4001-16D | 40 | 60 |
| 4001-17D | 100 | 100 |
| 4001-18D | 87 | 100 |

*% of 15 colonies tested that were agglutinated 3+ or 4+ by antibody to type 1 somatic pili of *E. coli* H10407.

TABLE 5

RESPONSE OF VACCINEES IMMUNIZED WITH TWO PARENTERAL DOSES OF PURIFIED E. COLI TYPE 1 SOMATIC PILI VACCINE AND CONTROLS FOLLOWING INGESTION OF $5X10^8$ VIRULENT ENTEROTOXIGENIC E. COLI (STRAIN H10407)

| Group | Mean Incubation (hrs) | Diarrhea | Mean Total Diarrheal Stool Volume per Ill Volunteer | Mean Total No. Loose Stools per Ill Volunteer | Vomiting | Malaise | Fever | Positiv Stool Culture |
|---|---|---|---|---|---|---|---|---|
| Vaccinees | 36.5 | 2/6* | 3.89+ (1.38-6.40)** | 16 (15-19)** | 0/6 | 0/6 | 0/6 | 6/6 |
| | | p=0.04 | | | | | | |
| Controls | 34.5 | 7/7 | 3.96 (1.37-9.86) | 18 (7-29) | 6/7 | 7/7 | 2/7 | 7/7 |

* No. positive/No. challenged
\+ liters
** (range)

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Appendix B

# SEROLOGIC RESPONSE IN VOLUNTEERS FOLLOWING PARENTERAL IMMUNIZATION WITH *ESCHERICHIA COLI* H10407 PURIFIED TYPE 1 SOMATIC PILI VACCINE AND FOLLOWING ORAL CHALLENGE WITH ENTEROTOXIGENIC *ESCHERICHIA COLI*

Charles C. Brinton, Jr.[1], Ph.D.,
Myron M. Levine[2], M.D., D.T.P.H.,
Roy Robins-Browne[2], M.B., B.Ch.,
Peter Fusco[1], Ph.D., Sarah Wood[1], Ph.D.,
Alfred Saah[2], M.D., Timothy P. Hughes[2], B.S., Charles R. Young[2], B.A.,
Sylvia O'Donnell[2], R.N., Malvi Solomon[2], M.D.,
Margaret B. Rennels[2], M.D.

Laboratory for Pilus Research and Development
Department of Biological Sciences
University of Pittsburgh,
Pittsburgh, Pennsylvania

Center for Vaccine Development,
Division of Infectious Diseases,
University of Maryland School of Medicine
Baltimore, Maryland 21201

## ABSTRACT

Purified Escherichia coli H10407 type 1 somatic pili vaccine was administered intramuscularly to adult volunteers. Circulating antibody to H10407 type 1 somatic pili, H10407 O antigen and heat-labile enterotoxin (LT) were measured in 21 volunteers who received primary immunization with 45 (3), 90 (4), 900 (4) or 1800 (10) mcg doses of vaccine, in 14 volunteers inoculated with an 1800 mcg booster inoculation one month after primary, and in six vaccinees and seven control volunteers who ingested $5X10^8$ E. coli H10407 during an experimental challenge study of vaccine efficacy. All 21 primary vaccinees developed significant rises in pili antibody measured by ELISA; antibody levels increased with vaccine dose. The GMT of persons who received 900 or 1800 mcg primary doses did not rise following booster inoculation. In contrast, GMT of vaccinees who received 45 or 90 mcg primary doses rose four-fold (from 21 to 83), following booster inoculation. Pili vaccine did not stimulate rises in LT antitoxin but trace amounts of O antigen contained in the vaccine led to significant rises in O antibody in all 10 recipients of 1800 mcg primary doses and in one-half of those given lower doses. All controls developed significant rises in O and LT antibody following challenge. In the six vaccinees following challenge, LT antitoxin rose in four and O antibody in six. Rises in antibody to type 1 somatic pili failed to rise in either vaccinees or controls in response to virulent challenge.

## INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC), the most common cause of travelers' diarrhea[1,2] and an important source of diarrhea in infants,[3] piglets and calves,[4] adhere to small intestinal epithelial cells as a prerequisite to initiating toxin-mediated fluid secretion. Various classes of pili function as adhesive virulence factors enhancing the bacteria-host cell interaction. For instance, it has been shown that adhesion of ETEC to epithelial cells of pig small intestine occurs via the attachment of pili to specific epithelial cell receptors.[5,7] Pilus-specific antibody inhibits attachment and only serologically related pili competitively inhibit attachment.[5,7] A purified pilus vaccine given parenterally to pregnant sows provided significant protection against challenge with ETEC to piglets suckled on the immunized sows.[8] Immunity was conferred only against ETEC strains possessing the homologous pilus antigen and was correlated with selective inhibition of colonization of the small intestine by ETEC.[8]

The pili of ETEC strain H10407 (O78:H11), originally isolated from a case of severe diarrhea in Bangladesh, have been intensively studied by Brinton et al.[9,10] This organism produces type 1 somatic pili (which cause mannose-sensitive hemagglutination of guinea pig erythrocytes)[11,12] and another class of pili (NMS) that manifests non-mannose sensitive agglutination of human type A or B erythrocytes.[9,13] The NMS pili are probably identical to the so-called colonization factor antigen/I described by Evans et al.[14] Type 1 somatic pili of E. coli H10407 are controlled by chromosomal genes[10] and NMS pili apparently by plasmid genes.[15] Each type of pilus appears to have independent systems for phenotypic phase variation and quantitative control of pilus expression. By varying the culture medium and growth conditions, E. coli H10407 can be cloned to yield cultures in which bacterial cells contain both pilus classes, or only one or the other. This has led to purification of the type 1 somatic and NMS

pili for evaluation as vaccines in man.

In this paper we describe the circulating antibody response following parenteral inoculation of volunteers with purified type 1 somatic pili from E. coli H10407 and following oral challenge with virulent E. coli H10407.

## MATERIALS AND METHODS

### Vaccine Preparation and Pre-testing:

The preparation and testing of the E. coli H10407 type 1 somatic pilus vaccine is described in detail elsewhere. Briefly, strain H10407 was cloned according to colonial type and grown under conditions in which only its type 1 pili were expressed. After harvesting the growth from solid medium in a buffer in which the pilus rods were soluble the suspension was blended and bacteria were removed by centrifugation. Three to five cycles of pilus crystal solubilization and recrystallization were sufficient to prepare concentrated type 1 pili which were pure by our standard criteria. The pilus suspensions were sterilized by membrane filtration in soluble (single-rod) form. Merthiolate was added as a preservative before filtration. The concentration of the vaccine was 1.8 mg/ml of pilus protein. The vaccine was tested for general safety, sterility and product safety according to standard procedures recommended by the Bureau of Biologics of the Food and Drug Administration and was approved for experimental use as a parenteral vaccine.

### Antibody Assays

### Antipili Antibody

Type 1 pili antibodies in serum were measured by enzyme-linked immunosorbent assay (ELISA) developed by Brinton et al[16] as reported elsewhere. Briefly, plastic cuvettes were coated with purified type 1 somatic pili derived from strain H10407. The serum to be assayed was added to the coated cuvette and incubated; it was then rinsed to remove unbound antibody. Peroxidase-conjugated goat anti-human IgG was then added to the cuvettes and incubated. Unbound conjugate was removed

with rinsing and substrate (peroxide) and indicator dye (O-dianisidine) were added. After appropriate incubation the reaction was stopped by the addition of acid and the absorbance was read in a Gilford Stasar II clinical spectrophotometer.

Pili antibody levels were standardized using a high titer standard serum from a volunteer who was immunized with two parenteral inoculations of purified H10407 type 1 somatic pili vaccine. Serial dilutions of the standard serum were tested in every series of assays and a standard curve was constructed. Antibody levels of unknown sera were determined from the standard curve relative to an arbitrary level of 56,000 assigned to the standard serum. Sera were assayed in duplicate. The reproducibility of the average of duplicate assays in this system is on the order of 15% from run-to-run, day-to-day, and month-to-month.

Antibody to Heat-Labile Enterotoxin:

Antibody to E. coli heat-labile enterotoxin was measured by the Y-1 adrenal cell neutralization assay as previously described.[17,18]

Antibody to O Antigens:

Antibody to the somatic O antigens of E. coli H10407 and B7A (O148:H28) was assayed by microtiter passive hemagglutination technique as previously described.[18] Alkaline extract lipopolysaccharide (O) antigen was prepared by the method of Young et al[19] from a clone of E. coli H10407 lacking both type 1 somatic and NMS pili. In the same manner lipopolysaccharide O antigen was prepared from a piliated clone of E. coli B7A[18] (which possesses type 1 somatic pili antigenically identical to those of E. coli H10407). One ml. of a 10% suspension of glutaraldehyde-treated[20] sheep erythrocytes was coated with 0.1 ml of antigen. After heat inactivation (56°C, 30 min.) and absorption with unsensitized sheep erythrocytes, anti-O antibody was measured by microtiter passive hemagglutination

technique.[18,21] Two-fold dilutions of sera (0.025 ml) were added to microtiter wells followed by 0.025 ml of a 0.5% suspension of the sensitized sheep erythrocytes. Plates were incubated for 2 hrs. at 37°C and 16 hr. at 4°C before they were read. High titer lapine antisera from rabbits immunized with H10407 and B7A served as positive controls.

Sera

Twenty-one healthy adult volunteers were given a primary intramuscular (IM) immunization with 45, 90, 900 or 1800 mcg of purified pili vaccine. Serum specimens were obtained pre-immunization and 10, 21 and 28 days post-immunization.

Fifteen volunteers received an 1800 mcg. IM booster immunization 28 days after the first dose of vaccine. Sera were obtained 10, 21 and 28 days after the booster from 14 vaccinees.

Sera were also tested from six immunized and 7 unimmunized control volunteers who participated in a challenge study of vaccine efficacy. Sera were obtained pre- and 10, 21 and 28 days post-challenge.

RESULTS

Immunogenicity Studies

Antibody to Type 1 Somatic Pili

Antibody to type 1 somatic pili in volunteers before and after immunization are seen in Table 1. All 21 immunized persons developed four-fold or greater rises following primary immunization, including the 7 volunteers who received low doses of 45 or 90 mcg. of purified pili vaccine.

The kinetics of the immune response, relation to antigenic load and the effect of booster immunization are seen in Figure 1. Although antibody rises were evident by day +10, levels peaked on day 28 after the first dose of vaccine. Antibody levels attained following primary immunization were related to antigenic load. The GMT on day 28 of vaccinees who received 900 or

1800 mcg. (122) was significantly higher than the GMT (21) of those who received 45 or 90 mcgs. By 28 days after booster inoculation of the low dose group with an 1800 mcg. immunization (56 days after primary dose), the GMT rose four-fold to a titer of 82. In the group who received 900 or 1800 mcg. primary doses, the 1800 mcg booster inoculation did not stimulate a further rise in GMT between day 28 (164) and day 56 (159) Figure 1.

The relationship between antigenic mass and antibody response on day 28 following a single dose of vaccine is seen in Table 2.

Antibody to LT:

No significant rises in LT antitoxin occurred following primary or booster immunization with pili vaccine.

Antibody to Somatic O Antigen:

Two of seven volunteers who received 45 or 90 mcg. doses of vaccine had four-fold rises in antibody to O antigen of E. coli H10407. Significant rises in H10407 O antibody also occurred in two of four volunteers who received 900 mcg. and in all 10 who were inoculated with 1800 mcg. primary doses of vaccine (Table 3). In all but one instance antibody peaked on day 10. Titers were significantly higher in recipients of the 1800 mcg dose. In contrast, none of the 21 vaccinees developed four-fold or greater rises to the heterologous O antigen (O148) of E. coli B7A.

Challenge Studies

Antibody to Type 1 Somatic Pili

Approximately eight weeks after initial parenteral immunization with pili vaccine, six vaccinees and seven unimmunized control volunteers participated in a challenge study of vaccine efficacy wherein they ingested $5X10^8$ virulent organisms of E. coli H10407. Clinical results of this challenge are described in detail in the accompanying paper.[22] Two vaccinees and all seven controls

were centrifuged, the supernatants discarded and the bacterial pellets tested for agglutination with antiserum to purified H10407 type 1 somatic pili; the proportion of cultures reacting with specific antibody were recorded.

During Challenge:

Stool specimens or rectal swabs were collected daily throughout the challenge study (including at least two pre-challenge specimens) and inoculated onto Levine's EMB agar. Ten colonies with a typical E. coli metallic sheen were sub-cultured onto slants of trypticase soy agar in screw-top tubes. After 18 hrs. of incubation at 37°C these clones were tested for agglutination with rabbit antisera to E. coli H10407, H10407 type 1 somatic pili and H10407 NMS pili. The isolates were also tested for LT production by Y-1 adrenal cell assay as previously described.[34,35]

RESULTS:

Reactogenicity:

Clinical:

Neither erythema, induration, heat, tenderness, fever nor malaise occurred in any of the 21 volunteers who received primary immunization with varying dose of purified pili vaccine (Table 1). Among the fifteen persons who received an 1800 mcg booster inoculation no systemic adverse reactions were noted but six vaccinees developed objective local adverse reactions including induration, heat or erythema (Table 2). Local reaction following the booster occurred in persons who had received primary inoculations with 45 (2), 900 (2) and 1800 (2) mcg doses of vaccine. Local reactions were evident 24 hrs post-inoculation but, with one exception, were gone by 48 hrs. The reactions were described as mild to moderate by the volunteers. In no instance did nausea, vomiting, diarrhea or fever occur.

Gastrointestinal Function:

Approximately two-thirds of the vaccinees had measurements of intestinal

those receiving lower dose primary immunizations), developed significant rises in antibody to H10407 O antigen. The titers peaked on day 10 and fell rapidly thereafter, typifying the prodominantly IgM class antibody response to O antigen.[23] We are confident that the antibody measured by PHA was indeed to H10407 O antigen and not to type 1 pili that may have contaminated the O antigen preparation. To preclude such confusion, H10407 O antigen was deliberately prepared from a clone devoid of both type 1 somatic and NMS pili. Furthermore, no rises were recorded when sera were tested against an O antigen prepared from E. coli B7A which has an O antigen serologically distinct from H10407 (O148 vs. O78) but possesses antigenically identical type 1 somatic pili. The appearance of antibodies to H10407 O antigen following immunization with "high" doses of pili vaccine, therefore, must represent contamination of the vaccine preparation with minute quantities of O antigen. Studies by Greisman et al. have shown that man will develop significant rises in circulating antibody in response to minute parenteral doses of E. coli O lipolysaccharide (0.001-0.01 mcg/kg) if the immunizing preparation also contains protein.[24]

By the time of challenge with virulent ETEC H10407 the short-lived circulating IgM anti-O titers had dropped and approximated pre-immunization titers. In contrast, long-lived IgG class anti-pili antibody remained at high levels. Following challenge, both unimmunized control volunteers and vaccinees showed prominent circulating anti-O and anti-LT responses. However, neither vaccinees nor controls manifested significant rises in titer of circulating antibody to type 1 somatic pili. The reasons for this lack of immune response to this antigen are unclear. It is possible that a secretory IgA response occurred in the intestinal mucosa which was not detected by measurement of circulating IgG anti-pili antibodies.

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Table 1

CIRCULATING IgG ANTI-TYPE 1 PILI ANTIBODY IN RECIPIENTS OF PURIFIED TYPE 1 PILI VACCINE

| | | Days Post-Immunization | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Vaccinee | Priming Vaccine Dose | 0 | 10 | 21 | 28 | Booster Vaccine Dose | 39 | 49 | 56 |
| 4001-1 | 45* | 2† | 17 | 33 | 25 | 1800* | 38 | NA | 78 |
| 4001-2 | 45 | 3 | 12 | 23 | 26 | 1800 | 89 | 219 | 237 |
| 4001-3 | 45 | 2 | 10 | 27 | 27 | 1800 | 48 | 81 | 100 |
| 4001-5 | 90 | 1 | 24 | 34 | 29 | - | NA | NA | NA |
| 4001-6 | 90 | 1 | 6 | 8 | 8 | 1800 | 21 | 54 | 58 |
| 4001-7 | 90 | 1 | 14 | 11 | 11 | 1800 | 16 | 26 | 36 |
| 4001-8 | 90 | 1 | 62 | 43 | 44 | 1800 | 33 | 58 | 81 |
| 4001-9 | 900 | 2 | 8 | 90 | 79 | - | NA | NA | NA |
| 4001-10 | 900 | 2 | 36 | 64 | 134 | 1800 | 218 | 450 | 286 |
| 4001-11 | 900 | 2 | 125 | 225 | 155 | 1800 | 107 | 103 | 113 |
| 4001-12 | 900 | 8 | 20 | 189 | 328 | 1800 | NA | NA | 384 |
| 4001-13 | 1800 | 1 | 172 | 181 | 208 | 1800 | NA | NA | NA |
| 4001-14 | 1800 | 3 | 304 | 377 | 512 | 1800 | 332 | 539 | 546 |
| 4001-15 | 1800 | 2 | 43 | 70 | 64 | 1800 | 63 | 64 | 71 |
| 4001-16 | 1800 | 2 | 71 | 60 | 69 | 1800 | 46 | 56 | 47 |
| 4001-17 | 1800 | 5 | 473 | 415 | 423 | 1800 | 280 | NA | 415 |
| 4001-18 | 1800 | 3 | 86 | 96 | 82 | 1800 | 67 | 68 | 43 |
| 4001-19 | 1800 | 2 | NA | 82 | 79 | - | NA | NA | NA |
| 4001-20 | 1800 | 2 | 47 | 72 | 42 | - | NA | NA | NA |
| 4001-21 | 1800 | 1 | 31 | 39 | 29 | - | NA | NA | NA |
| 4001-23 | 1800 | 2 | 188 | 203 | 189 | - | NA | NA | NA |

*mcg of purified pili

†reciprocal ELISA titer $X10^{-3}$

Table 2

CIRCULATING IgG ANTI-PILI ANTIBODY IN VOLUNTEERS GIVEN PARENTERAL PRIMARY IMMUNIZATION WITH VARYING DOSES OF PURIFIED TYPE 1 SOMATIC PILI VACCINE FROM E. COLI H10407

| | DAY | | | |
|---|---|---|---|---|
| Vaccine Dose | 0 | 10 | 21 | 28 |
| 45 or 90 mcg | 2* | 16 | 22 | 21 |
| N | 7 | 7 | 7 | 7 |
| 900 or 1800 mcg | 2 | 72 | 120 | 122 |
| N | 14 | 13 | 14 | 14 |

*reciprocal ELISA titer $X10^{-3}$

Table 3

ANTI-O ANTIBODY RESPONSE FOLLOWING PRIMARY IMMUNIZATION WITH VARYING DOSES OF *E. COLI* H10407 PURIFIED SOMATIC TYPE 1 PILI VACCINE.

| Vaccine Group | No. with Significant Antibody Rises*/No. Immunized | Reciprocal Geometric Mean Titer† | | | |
|---|---|---|---|---|---|
| | | Day:0 | +10 | +21 | +28 |
| 45 or 90 mcg. | 2/7 | 8.8 | 23.8 | 17.7 | 13.1 |
| 900 | 2/4 | 4.8 | 19 | 13.5 | 11.3 |
| 1800 | 10/10 | 2.6 | 276 | 111 | 64 |

*Four-fold or >

†Measured by passive hemagglutination

Table 4

Appendix B

LEVELS OF CIRCULATING ANTIBODY TO TYPE 1 SOMATIC PILI MEASURED BY ELISA IN VACCINEES AND CONTROLS CHALLENGED WITH *E. COLI* H10407

| | | DAYS POST-CHALLENGE | | | |
|---|---|---|---|---|---|
| Vaccinees | Clinical Illness | 0 | 10 | 21 | 28 |
| 4002-2 | + | 41 | 57 | 43 | 46 |
| 4002-5 | - | 47 | 55 | 70 | 45 |
| 4002-10 | - | 25 | 47 | 38 | 39 |
| 4002-12 | - | 342 | 352 | 376 | 443 |
| 4002-14 | + | 84 | 47 | 54 | 77 |
| 4002-18 | - | 263 | 306 | NA | 336 |
| GMT† | | 84 | 95 | 75 | 99 |
| Controls | | | | | |
| 4002-1 | + | 1 | 1 | 1 | 1 |
| 4002-3 | + | 1 | 1 | 1 | 1 |
| 4002-4 | + | 1 | 1 | 2 | 2 |
| 4002-9 | + | 1 | 1 | 1 | 1 |
| 4002-11 | + | 2 | 2 | 2 | 2 |
| 4002-13 | + | 1 | 1 | 1 | 1 |
| 4002-16 | + | 1 | 2 | 2 | 2 |
| GMT | | 1.0 | 1 | 1 | 1 |

*reciprocal ELISA titer $X10^{-3}$ †reciprocal geometric mean titer

Table 5

SERUM LT ANTITOXIN IN VACCINEES AND CONTROLS FOLLOWING CHALLENGE WITH $10^8$ *E. COLI* H10407.

| Vaccinees | Clinical Illness | Days Post-Challenge 0 | 10 | 21 | 28 |
|---|---|---|---|---|---|
| 4002-2 | + | 4* | 64 | 256 | 128 |
| 4002-5 | - | <4 | <4 | <4 | <4 |
| 4002-10 | - | 128 | 512 | 512 | 256 |
| 4002-12 | - | <4 | <4 | <4 | <4 |
| 4002-14 | + | 32 | 128 | 128 | 16 |
| 4002-18 | - | <4 | 16 | NA | 32 |
| GMT† | | 7 | 25.4 | 36.8 | 20.2 |
| Controls | | | | | |
| 4002-1 | + | <4 | <4 | 128 | 64 |
| 4002-3 | + | <4 | 256 | 128 | 128 |
| 4002-4 | + | <4 | <4 | <4 | <4 |
| 4002-9 | + | <4 | 16 | 8 | 8 |
| 4002-11 | + | 16 | 2048 | 1024 | 256 |
| 4002-13 | + | <4 | 128 | 64 | 64 |
| 4002-16 | + | 4 | 32 | 16 | 32 |
| GMT† | | 3.0 | 39 | 43 | 35.3 |

*antitoxin units/ml of serum

†reciprocal geometric mean titer

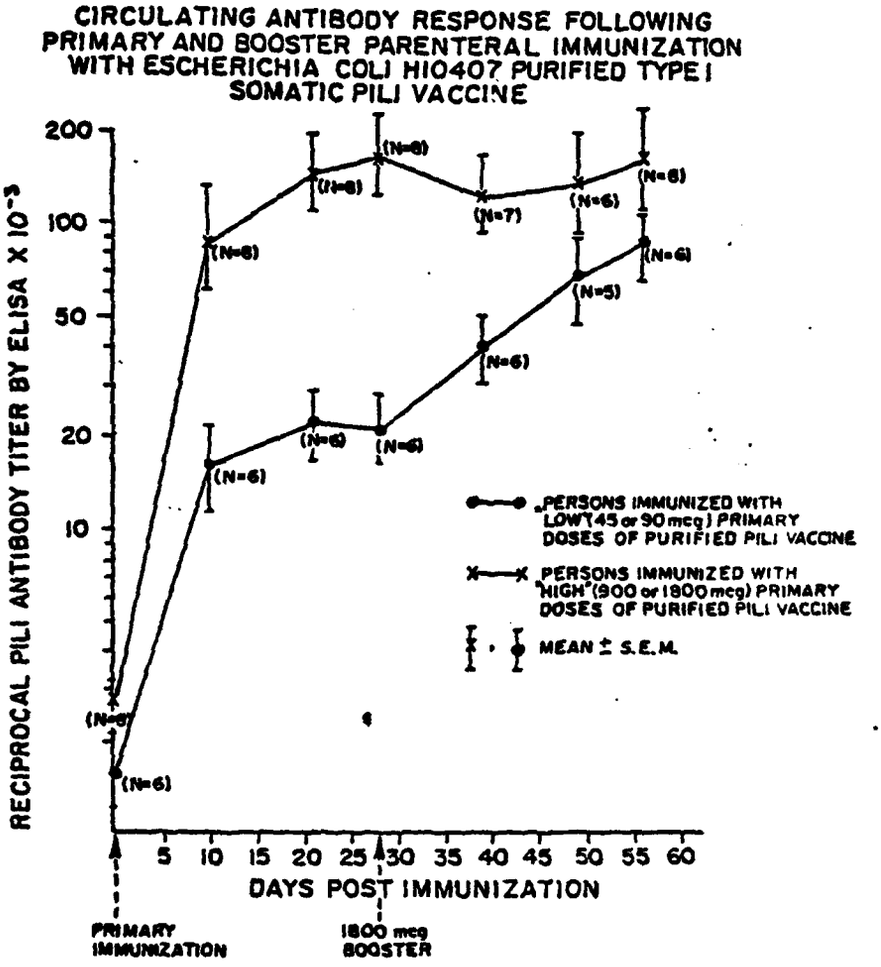
ANTIBODY TO O ANTIGEN OF E. COLI H10407 IN VACCINEES AND CONTROL VOLUNTEERS FOLLOWING CHALLENGE WITH $5X10^8$ ENTEROTOXIGENIC E. COLI H10407 ORGANISMS

| Vaccinees | Clinical Illness | Days Post-Challenge 0 | 10 | 21 | 28 |
|---|---|---|---|---|---|
| 4002-2 | + | 32* | 256 | 64 | 32 |
| 4002-5 | - | 4 | 256 | 64 | 32 |
| 4002-10 | - | 8 | 8192 | 128 | 128 |
| 4002-12 | - | 8 | 256 | 16 | 16 |
| 4002-14 | + | 8 | 8192 | 256 | 64 |
| 4002-18 | - | 8 | 128 | NA | 32 |
| GMT† | | 9.0 | 724 | 74 | 40 |
| **Controls** | | | | | |
| 4002-1 | + | 2 | 8192 | 256 | 128 |
| 4002-3 | + | 2 | 2048 | 128 | 128 |
| 4002-4 | + | 2 | 4096 | 1024 | 128 |
| 4002-9 | + | 2 | 8192 | 128 | 128 |
| 4002-11 | + | 4 | 16,384 | 4096 | 4096 |
| 4002-13 | + | 8 | 8192 | 2048 | 256 |
| 4002-16 | + | <2 | 4096 | 128 | 64 |
| GMT† | | 2.4 | 6087 | 464 | 210 |

*Reciprocal hemagglutination titer.

†Reciprocal geometric mean titer.

FIGURE 1


CIRCULATING ANTIBODY RESPONSE FOLLOWING PRIMARY AND BOOSTER PARENTERAL IMMUNIZATION WITH ESCHERICHIA COLI H10407 PURIFIED TYPE I SOMATIC PILI VACCINE
RECIPROCAL PILI ANTIBODY TITER BY ELISA X $10^{-3}$
200
100
50
20
10
(N=6)
(N=7)
(N=5)
PERSONS IMMUNIZED WITH LOW (45 or 90 mcg) PRIMARY DOSES OF PURIFIED PILI VACCINE
PERSONS IMMUNIZED WITH "HIGH" (900 or 1800 mcg) PRIMARY DOSES OF PURIFIED PILI VACCINE
MEAN ± S.E.M.
5 10 15 20 25 30 35 40 45 50 55 60
DAYS POST IMMUNIZATION
PRIMARY IMMUNIZATION
1800 mcg BOOSTER

END

FILMED

9-83

DTIC